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EFFECTS OF ANGIOTENSIN II TYPE 1 RECEPTOR ANTAGONISTS ON MYOCARDIAL ISCHEMIA/REPERFUSION INJURY

BY

ROHIT MOUDGIL

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled EFFECTS OF ANGIOTENSIN II TYPE 1 RECEPTOR ANTAGONISTS ON MYOCARDIAL ISCHEMIA/ REPERFUSION INJURY, submitted by ROHIT MOUDGIL in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in EXPERIMENTAL MEDICINE.

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ABSTRACT

Effects of angiotensin II type 1 receptor (AT_1R) antagonists candesartan (CAN) and losartan on functional recovery after ischemia-reperfusion (IR) and angiotensin II type 2 receptor (AT_2R) protein modulation were studied in isolated rat hearts. In the Langendorff system, CAN pretreatment for 40 min improved functional recovery after IR (30 min of ischemia; 40 min of reperfusion) and increased AT_2R protein without any detectable change in apoptosis.

In isolated working hearts, CAN for 40 min before and during ischemia (25 min) also improved post-ischemic functional recovery. Although losartan did not significantly improved in post-ischemic contractile recovery, both losartan and CAN, increased AT_2R protein content, myocardial cGMP content and PKC_ε protein. The AT_2R antagonist PD123319, when co-administered with CAN attenuated the observed functional and molecular effects of CAN.

Cumulatively, these data suggest that CAN-induced cardioprotection is associated with unopposed AT_2R activation and that AT_2R-mediated cardioprotection involves cGMP increase and PKC_ε activation.

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List of Abbreviations

ACEI	Angiotensin Converting Enzyme Inhibitors
ANF	Atrial Natriuretic Factor
Ang II	Angiotensin II
AP1	Activating Protein 1
AT_1R	Angiotensin II Type 1 Receptor
AT_2R	Angiotensin II Type 2 Receptor
CABG	Coronary Artery Bypass Graft
cAMP	Cyclic Adenosine 3′, 5′ Monophosphate
cGMP	Cyclic Guanosine 3′, 5′ Monophosphate
CF	Coronary Flow
CAN	Candesartan
CRE	Cyclic AMP-Responsive Element
DAG	Diacylglycerol
GRE	Glucocorticoid-Responsive Element
IL-1β	Interleukin 1β
IP_3	Inositol 1,4,5 Trisphosphate
IPC	Ischemic Preconditioning
IR	Ischemia-Reperfusion

Jak	Janus Kinase
K_{ATP}	ATP-sensitive K^+ Channel
LVEDP	Left Ventricular End Diastolic Pressure
LVSP	Left Ventricular Systolic Pressure
LVDP	Left Ventricular Developed Pressure
MAPK	Mitogen Activated Protein Kinase
α, β MHC	Myosin Heavy Chain α and β isotype
MI	Myocardial Infarction
MKP-1	Mitogen Activated Protein Kinase Phosphatase-1
NFκB	Nuclear Factor kappa B
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
PC	Preconditioning
PD	PD123319 (AT_2R Antagonist)
PI3K	Phosphotidinositol-3-Kinase
PKC_α	Protein Kinase C-alpha isoform
PKC_ϵ	Protein Kinase C-epsilon isoform
PTP	Phosphotyrosine Phosphatases
PTCA	Percutaneous Transluminal Coronary Angioplasty

RAS	Renin Angiotensin System
STAT	Signal Transducer and Activators of Transcription
TUNEL	Terminal Deoxynucleotidyl Transferase [Tdt] Mediated dUTP Nick End Labeling
Tyk	Tyrosine Kinase
VSMCs	Vascular Smooth Muscle Cells

INTRODUCTION

Angiotensin II (Ang II) is an octapeptide that has been a subject of a great deal of interest, and the list of its biochemical, physiological and pathophysiological actions are extensive and still growing. The ongoing history and progression of our understanding of the renin-angiotensin system (RAS) has been one of the most fascinating aspects of modern biology and medicine. It was in 1898 that Tigerstedt and Bergmann [4] isolated a pressor substance from renal extracts, which marked the beginning of research on this previously unidentified system. However, it was not until 1934 that Goldblatt [2] confirmed the source of pressor substance by constricting one of the renal arteries. These observations, in addition to other studies [4], led to the identification of RAS and subsequent implication in the reno-vascular hypertension. Later, when the importance of Ang II (Figure 1) in the control of mineralocorticoid metabolism and thirst became clarified, it became evident that the renal RAS plays an integral role in the maintenance of fluid and electrolyte homeostasis throughout the body [3].

The past decade has witnessed the emergence of a new role for the RAS in directly controlling cardiac function. The heart is among a number of organs that have their own intrinsic RAS. Due to the potential implications of the local synthesis and actions of Ang II in the heart, a great deal of interest and enthusiasm has been generated throughout the scientific community to elucidate the very nature of the intracardiac tissue RAS.

Today, it is widely recognized that persistent activation of the Ang II-mediated pathway precipitates various cardiovascular pathologies. Ailments such as hypertension,

coronary artery disease, myocarditis, congestive heart failure and myocardial infarction involve Ang II action at both the local vascular and myocardial tissue levels [1].

Angiotensin II in the Heart

The RAS has been traditionally viewed as an endocrine system, where Ang II circulates in the blood and exerts its effects via Ang II receptors at different sites throughout the body (Table 1). Among its various actions, Ang II also mediates vascular smooth muscle constriction and thereby regulates vascular tone in conjunction with other vascular factors. However, during essential hypertension, the level of plasma Ang II is increased, precipitating a persistent constrictive state in the vasculature. To counteract this effect, angiotensin converting enzyme inhibitors (ACEI) were used as potential vasodilators, acting via inhibition of Ang II formation. Surprisingly, ACEI, in contrast to other vasodilators, prevented and reversed the cardiac hypertrophy associated with hypertension, suggesting a trophic role of Ang II in the heart [5,6]. Further studies have showed that subpressor doses of an ACEI, which do not reduce blood pressure or afterload, were able to regress cardiac hypertrophy [7,8]. Cumulative studies suggest that Ang II directly influence pathological cardiac hypertrophy, independent of a systemic effect.

In addition to the reduction of cardiac hypertrophy with subpressor doses in animals, patients receiving ACEI also showed some additional therapeutic benefits. In the acute stage of heart failure, reduction in cardiac output and increase in filling pressure result in the elevation of plasma renin activity, Ang II and aldosterone levels. This increase is, in turn, associated with vasoconstriction and sodium retention. However,

these circulating neurohumoral responses return to normal during the compensated stage of heart failure, as plasma volume and cardiac stroke volume increase. A similar normalization of plasma neurohumoral activity is also observed in mild and stable forms of myocardial infarction [9], and in animal models of compensated heart failure (i.e., coronary-ligated rats or subacute stage of canine rapid ventricular pacing) [10,11]. Under these pathological conditions of normal Ang II levels, long-term treatment with ACEI elicits salutary responses in patients and animal models, stabilizing cardiac recovery and decreasing overall morbidity and mortality [12-14]. These observations led to the hypothesis that perhaps locally but not systemically produced Ang II causes trophic effects on the heart. Subsequent studies designed to characterize local RAS led to the isolation of angiotensinogen [15], renin [16] and angiotensin converting enzyme[17] in various compartments of the heart. In addition, these factors are increased in different pathologies, thereby establishing the role of local Ang II in cardiac diseases [18].

Detailed analysis showed that locally produced Ang II induces its trophic effects on the myocardium by activating immediate-early genes. These factors act on the promoter regions of mRNA of various proteins to precipitate hypertrophy in cardiac myocytes and hyperplasia in fibroblasts [19]. Furthermore, Ang II also mediates activation and release of other factors (such as endothelin [20], transforming growth factor β_1 [21], and matrix metalloproteinase [22]) which cumulatively, affect the architecture of the heart. Despite the multitude of the actions displayed by Ang II, it seems to exert most of its effect by angiotensin II type 1 receptors.

Angiotensin II Type 1 Receptors (AT_1R)

Recent years saw a surge in concerted efforts to characterize the AT_1R. Since angiotensin II type 2 receptors (AT_2Rs) are not highly expressed in adult tissues (and thus none of the Ang II functions were originally attributed to it), initial experiments concentrated on and were geared towards identifying and isolating AT_1R. As a result of this research, a great deal of knowledge has been accumulated over the past decade. This has led to the development of selective, non-peptidic, orally effective antagonists of the AT_1R, which enabled the biochemical, physiological and pathophysiological aspects of this receptor to be defined and researched. The new knowledge has provided key insights about the role of Ang II in the pathophysiology associated with various cardiovascular diseases. However, further analysis of AT_1R properties is needed in order to determine its potential role in various cardiomyopathies.

Molecular Biology and Regulation of AT_1R

Existence of the AT_1R subtype was confirmed pharmacologically in 1989 [24]. However, it was the successful cloning of the AT_1R in 1991 that greatly facilitated further research on the structure and function of AT_1R [25,26] (Figure 2). AT_1Rs are distributed abundantly and ubiquitously throughout the heart, kidney, adrenal gland, liver, brain, lung and vascular tissues [4]. AT_1R mediates all the classical actions of Ang II including fluid volume homeostasis, regulation of vascular tone and release of other hormones. Recently AT_1Rs have also been implicated in pathogenesis of various cardiac diseases [4].

AT_1R belongs to a superfamily of G-protein coupled receptors that have seven transmembrane domains. AT_1R is a 359 amino acid protein with a calculated molecular

weight of the functional receptor as high as 79 kD [26]. Only a single gene encoding the AT_1R has been identified in humans, rabbits, and cows. Rodents, in contrast, have two highly homologous AT_1R genes that encode the receptor isotypes termed AT_{1A} and AT_{1B} (95% amino acid identity). While there are tissue-dependent differences in the expression pattern of the rodent AT_1 isotypes, both receptors bind Ang II and have intracellular signal transduction pathways that are identical [27].

Studies examining AT_1R regulation suggests modulation by three distinct pathways:

A) Internalization: Internalization is a process in which a receptor, engulfed by the cells, is recycled and this process, in this context, is initiated by binding of Ang II but not with available antagonists. Mutagenesis studies have shown that a carboxyl tail region of the rat $AT_{1A}R$ is the major internalization determinant as its deletion completely abolishes the internalization of bound ^{125}I-angiotensin II [28]. Subsequent studies suggested that AT_1R internalization occurs via clathrin-coated pits, like the other G-protein coupled receptors [29-31].

B) Signal transduction molecules: AT_1R mRNA can be regulated by a variety of signal transduction molecules, including cyclic adenosine monophosphate (cAMP). Infact, cAMP downregulates AT_1R mRNA, which is surprising considering that PKC, another important signal transduction molecule present downstream of AT_1R, does not have any effect.

C) Transcription factors: The 5′ flanking region of AT_1R also hosts binding sites for various immediate-early genes including cyclic AMP-responsive element (CRE), glucocorticoid-responsive element (GRE), activating protein 1 (AP1), and nuclear factor-

kappa B (NFκB)) all of which, upon binding, increase AT_1R mRNA expression [31,32]. These factors are implicated in potential increase in AT_1R population in cardiac hypertrophy [33] and therefore might involve direct transcriptional increase in AT_1R.

AT_1R Signaling

The AT_1R belongs to the G-protein superfamily of receptors, and is coupled to one of two heterotrimeric G-proteins: G_q and G_i. Binding of Ang II to AT_1R results in release of the α subunits of the G-protein, which stimulates phospholipase C and thus generates 1,4,5 inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 releases calcium ions (Ca^{2+}) from intracellular stores and DAG stimulates PKC, leading to an influx of extracellular Ca^{2+} from T-type calcium channels. These events cumulatively result in increased intracellular Ca^{2+}, which induces vasoconstriction [33-35].

Ang II also triggers mitogenic activity via AT_1R. PKC, as well as elevated intracellular Ca^{2+} levels, can promote the expression of growth-related inducible transcription factors such as c-fos, c-myc and c-jun [35-37]. Stimulation of AT_1R has been associated with the phosphorylation of a tyrosine residue on Janus Kinase 2 (Jak2) and tyrosine kinase 2 (Tyk2), which subsequently activates signal transducer and activators of transcription (STAT). This Jak-STAT pathway is responsible for transcripitional activation of early growth responses [38,39]. Other mitogen kinases such as mitogen activating protein kinase (MAPK) are also responsible for similar effects mediated by AT_1R [40]. Recent studies have added a new twist to the understanding of AT_1R signaling. Studies done by Zou *et al.*[41,42] have identified the molecular mechanisms by which Ang II displayed its hypertrophic and hyperplastic effects on cardiac myocytes and fibroblasts,

respectively. That study showed that in cardiac myocytes, where AT_1R couples to G_q, PKC (but not Ras) induces Raf-1-mediated MAPK activation. On the other hand, in cardiac fibroblasts in which AT_1R couples to G_i, Ras (but not PKC) activates MAPK. These results collectively suggest that when AT_1Rs of different cell types are activated by the same agonist (Ang II), distinct signal transduction pathways downstream from the receptors are activated (PKC or Ras), producing increased activity of the same effector molecule (MAPK). This study highlights one of the key characteristics of AT_1R; diversity of signal transduction pathways associated with the receptor even though the end-effector is identical. However, in situations of a persistently activated angiotensin system, increased Ang II precipitates uncontrolled mitogenesis, a pathophysiological feature of elevated Ang II levels.

Role of AT_1R in Pathophysiology of Cardiovascular Diseases

A great deal of evidence has established that Ang II causes hypertrophy of neonatal cardiac myocytes [19] and adult cardiac myocytes [43]. Ang II directly induces the fetal phenotype of gene expression, such as β-MHC, skeletal α-actin, and atrial naturetic factor (ANF) in neonatal rat cardiac myocytes, indicating the direct involvement of AT_1R in cardiac gene reprogramming *in vitro*. Furthermore, Ang II also stimulates the expression of immediate-early genes, including c-fos, c-jun, jun B, Egr-1 and c-myc [19] in cardiac myocytes. Induction of these fetal programs result in an abnormal growth of cardiac myocytes. Histologically, side-to-side slippage of myocytes has indicated early occurrence of myocyte hypertrophy, which progressively leads to increased ventricular volume and wall thickness in non-infarcted myocardium. This sets up a vicious cycle of

increased ventricular wall stress that, over time, fosters increased ventricular hypertrophy [44].

Similarly, Ang II also increases mRNA levels for c-fos, c-jun, jun B, Egr-1 and c-myc in cardiac fibroblasts. These effects also occur via AT_1R. Furthermore, Ang II also increases collagen, as well as mRNA expression and protein secretion of fibronectin and transforming growth factor β_1, respectively [45-47]. Cumulatively, these events result in a phenomenon referred to as fibrosis, which is characterized by an increase in cardiac fibroblast cell number and concomitant increase in collagen deposition. A continued accumulation of fibroblasts with collagen impairs diastolic activity and compromises systolic mechanics [18]. Over time, myocardial stiffness develops, which predisposes the heart to ventricular dysfunction. Collectively, fibrosis and cardiac hypertrophy change the morphology of the heart, an event often referred to as cardiac remodeling [48].

In other pathologies such as hypertension, left ventricular mRNA levels for skeletal α-actin, ANF and collagen type I and III are increased, while α-MHC mRNA levels are decreased in spontaneously hypertensive rats. However, treatment with AT_1R antagonists normalizes these phenotypic responses, implicating AT_1R in the pathophysiology of hypertension [48]. A similar hypertrophic phenotype also occurs during myocardial infarction (except for the lack of any change in α-MHC mRNA). Administration of AT_1R antagonists to coronary ligated rat hearts significantly suppresses cardiac hypertrophy, which is reflected by the corresponding prevention of collagen I and III mRNA increases at 1 and 4 weeks, respectively [50]. Moreover, recent studies have implicated Ang II in cardiomyopathy observed in diabetes [51,52].

Therefore, AT_1R plays an instrumental role in pathophysiology of various cardiac diseases. By virtue of its presence in different cell types and its connection with different second messenger systems, AT_1R can mediate a diverse array of responses ranging from vasoconstriction to a total remodeling of the heart.

Angiotensin II Type 2 Receptors (AT_2R)

Although the role of AT_1R has been thoroughly investigated, the roles of AT_2R both in normal and in various cardiac pathologies remain unclear. There is increasing evidence suggesting a cardioprotective role for AT_2R, thus implicating AT_2R as a potential therapeutic target for medical intervention. These findings have fueled a great deal of research into its biochemical, physiological and pathophysiological properties.

AT_2R is ubiquitously expressed throughout fetal tissue, suggesting a possible role of this receptor in fetal development and organ morphogenesis. AT_2R expression decreases rapidly after birth, and in the adult, expression of this receptor is limited mainly to the uterus, ovary, heart, adrenal medulla and certain brain nuclei [23,53]. However, recent studies have demonstrated a re-expression of AT_2R in various cardiac pathologies, where in some cases, AT_2R becomes the dominant receptor subtype for Ang II in the myocardium [53].

Molecular Biology and Regulation of AT_2R

Preliminary findings suggested that AT_2R was not a seven transmembrane receptor (Figure 3), given that its ligand binding affinity was not reduced by stable GTPγS analogs, and it did not show agonist-induced internalization (features normally seen only

in seven transmembrane G-protein coupled receptors). However, subsequent expression cloning of cDNA isolated from PC12W cells [86] and rat fetus [87] encoded a 363 amino acid protein with seven transmembrane domains, suggesting the possibility of AT_2R being coupled to a G-protein. This was confirmed by Zhang and Pratt, who reported the immunoprecipitation of AT_2R with $G_{i\alpha 2}$ and $G_{i\alpha 3}$ [88].

AT_2R represents a special class of G-protein coupled receptors, as only a few of the seven transmembrane domain receptors have been reported to be GTPγS insensitive [55] (others are somatostatin [56] and dopamine D_3 [57]). Besides GTPγS-insensitivity, AT_2R, somatostatin and dopamine D_3 share a common, well-conserved third cytosolic loop as a major site for G-protein interaction [54-56]. AT_2R is unique in that it activates certain phosphotyrosine phosphatases (PTP), which is in contrast to somatostatin and D3 receptors which inhibits PTP [58,59]. Overall, AT_2R is a unique receptor, occupying a distinct position among the seven transmembrane domain family of receptors.

AT_2R is modulated by a variety of neurohumoral, cellular, and pharmacological factors. It is downregulated by gluocorticoids, growth factors, phorbol esters, calcium ionophores, G_q-coupled receptor stimulation, norepinephrine and cAMP. In contrast, insulin, insulin-like growth factor and cytokines upregulate AT_2R protein expression. Among these, calcium ionophores and norepinephrine represent some of the most the potent regulators of AT_2R expression [23]. Although most of the above mentioned factors regulate AT_2R post-transcriptionally, glucocorticoids and cAMP analogs can regulate AT_2R at the gene transcription level [23]. Recent evidence also suggests that Ang II-AT_2R binding enhances AT_2R levels in R3T3 cells [60]. The study suggests that when ligands bind to AT_2R, it forms a stable complex such that degradation of AT_2R is inhibited. This

phenomenon has been observed with different cell culture studies. However, the pathway involved in this phenomenon has not been explored yet.

AT_2R Signaling

Before the structure of AT_2R was determined, numerous attempts were made to elucidate the signal transduction pathways associated with this receptor subtype. Cell lines such as pheochromcytoma (PC12W), neuroblastoma (NG108-15, N1E-115) and fibroblast (R3T3), as well as primary cultures of neurons and cardiac myocytes, were used as models for signaling studies, which sometimes led to disparate theories [23,53]. However, recent studies have unraveled some of the pathways behind AT_2R signaling [53].

Inhibition of Mitogenesis

Stimulation of AT_2R activates PTP, which inhibits cell proliferation and differentiation [61,62]. This AT_2R-mediated PTP activation has been shown to inhibit AT_1R-mediated cell growth in myocytes and fibroblasts isolated from neonatal rat hearts [63], cardiomyopathic hamster hearts [64] and mouse hearts overexpressing AT_2R. The mechanism of AT_2R participation in anti-growth might also be associated with reduced MAPK activity caused by the activation of MAPK phosphatases-1 (MKP-1). However, other PTPs are also involved [66,67]. Nevertheless, an inhibitory effect on mitogenesis by AT_2R is achieved. Besides, its role in inhibition of MAPK, MKP-1 has also been implicated in cellular apoptosis.

Apoptosis

Apoptosis is a morphologically distinct mode of cell death whereby a cell commits suicide [69]. Apoptosis is a cell autonomous mechanism to eliminate injured or unwanted cells without inducing an inflammatory response. This is in contrast to necrosis, another mode of cell death, which results from an externally derived insult and which has a tendency to precipitate inflammation. Although apoptosis is a physiological process, excessive apoptosis has been linked to organ atrophy and failure [70]. Of relevance to the cardiovascular system, this excessive apoptosis has been implicated in myocardial infarction, heart failure, atherosclerosis (as reviewed by Haunstetter and Izumo [70]) and recently in IR [71].

Among the potential factors that induce apoptosis (such as atrial naturetic factor and hypoxia), Ang II has been implicated with apoptosis in cardiovascular diseases [69]. Studies looking at AT_2R transfected VSMCs showed that selective AT_2R stimulation enhanced apoptosis after serum starvation [71]. In addition, it has been demonstrated that AT_2R exerts a proapoptotic effect in neonatal cardiomyocytes, PC12W cells, and R3T3 mouse fibroblasts [66,71,72]. Furthermore, Li *et al.* demonstrated that Ang II induces apoptosis in skin fibroblasts of the mouse embryo but not in those prepared from AT_2R knockout mice, thereby implicating AT_2R as an inducer of apoptosis [76]. Some of the mechanisms proposed for AT_2R-induced apoptosis includes MKP-1-mediated dephosphorylation and subsequent inactivation of antiapoptotic factor, Bcl_2, increase expression of proapoptotic factor, bax and stimulation of *de novo* ceramide (a factor involved in inducing apoptosis) production [53,75].

In contrast, evidence also suggests that AT_1R might also be an inducer of apoptosis. Kajstura and colleagues [77] have reported that Ang II induced apoptosis in cultured rat neonatal ventricular myocytes through AT_1R-mediated PKC activation, an effect blocked by the AT_1R antagonist, losartan. Furthermore, p53, a gene associated with DNA-damaging agents, has been shown to increase angiotensinogen and AT_1R mRNA expression, which has been suggested to increase the susceptibility of cardiomyocytes to undergo apoptosis [78, 83]. Although, little additional evidence in support of AT_1R-induced apoptosis exists, further research is clearly warranted to resolve the issue of which receptor subtype and to what extent each receptor subtype contributes to apoptosis in specific cell types.

AT_2R Mediated Vasodilation

Studies in microvessels from canine coronary arteries [79], rat aortic strips [80], rat kidneys [81] and rat hearts [82] have shown that AT_2R activation results in formation of kinins and elevation of cGMP. Specifically, studies in blood vessels have shown that AT_2R activation directly increases nitric oxide (NO) as indicated by increase in nitrite and nitrate levels. This increase in NO was abolished by a bradykinin B_2 receptor, antagonist signifying bradykinin plays a role upstream of NO [79]. Subsequent studies showed that AT_2R activation resulted in an increased cGMP level which was abolished by a B_2 antagonist as well as by a nitric oxide synthase (NOS) inhibitor [82]. Taken together, evidence suggests that when AT_2R is stimulated, kinin is produced which subsequently leads to NO-mediated increased cGMP levels.

Recent investigations have partially outlined the cause behind the observed increase in kinin levels, cGMP, and nitrite levels by AT_2R [79, 101]. Tsutumi *et al.*[85] showed that AT_2R stimulation causes intracellular acidosis through the inhibition of Na^+/H^+ exchanger, resulting in enhancement of kininogenase activity in VSMCs. This in turn generates bradykinin, which acts on the endothelial bradykinin receptor in a paracrine manner to cause endothelium-dependent NO release. This leads to increased cGMP content and associated vasodilation.

Beside cGMP-dependent vasodilation, recent evidence also suggested AT_2R mediates cytochrome P450 dependent vasodilation (independent of cGMP). This study showed that activation of the AT_2R in afferent arterioles causes endothelium-dependent vasodilation via the cytochrome P-450 pathway, possibly by epoxyeicoatrienoic acids [89].

Studies with avian cardiomyocytes have shown that AT_2R contributes to the activation of IP_3 [90] and PKC [84]. However, the avian origin of the tissue restricts the extrapolation of the findings to mammalian tissues. AT_2R is also involved in activation of phosphotideinositol-3 kinase (PI3K), Na^+/HCO_3^- and release of arachidonic acid, although, the confirmation of these AT_2R functions is still necessary [91]. AT_2R has been reported to elicit stimulation of outward [92] and delayed rectifier potassium current [93] as well as inhibition of T-type calcium current [94].

Thus, efforts to investigate of the role of AT_2R subtype in Ang II-mediated effects have been hampered by lack of knowledge concerning the properties of AT_2R. The development of selective agonists and antagonists has enabled scientists to delineate some of the signaling pathways of AT_2R. However, the role of these second messenger

systems in physiological and pathophysiological settings clearly warrants further research.

Role of AT_2R in Pathophysiology of Cardiovascular Diseases

Earlier studies showed that AT_2R is expressed in neonatal hearts as well as in diseased hearts. Cellular localization of AT_2R in the human heart showed that AT_2R is located primarily in fibroblasts. Furthermore, expression of AT_2R in the heart increases concomitantly with the progression of intersitial fibrosis implicating AT_2R expression increase during cardiac remodeling in human hearts [95, 96]. AT_2R expression is also increased in failing hearts, although the precise mechanism of AT_2R expression and its role in diseased state has not yet been clarified [97]. It has been postulated that perhaps IL-1β causes the re-expression of AT_2R by binding to the promoter region of its sequence [97].

Functionally, AT_2R may play a role in various cardiac pathologies. AT_1R blockade may shift cardiac Ang II towards AT_2R causing growth inhibition as observed in proliferating rat coronary endothelial cells [98], transfected vascular smooth muscle cells (VSMCs) [99] and in hypertensive rats [101]. It was postulated that the antigrowth effects mediated by AT_2R might involve an increase in apoptosis [53]. However, activating apoptosis may also have detrimental consequences as excessive apoptosis has been implicated in organ atrophy and failure [69]. On the other end of the spectrum, scientists have also suggested protective effects with AT_2R [76, 100].

AT_2R increases kinins and thereby increases NO biosynthesis. Some of the cardioprotective actions attributed to AT_2R activation might include NO-induced cGMP mediated myocardial actions. These include (1) modulation of sarcolemmal Ca^{2+} influx,

(2) reduction in myofilament Ca^{2+} sensitivity, (3) altered SR function, (4) changes in the action potential, (5) modulation of cell volume, and (6) reduction in oxygen consumption [120].

Recently, another downstream pathway of NO has been elucidated. Ping *et al.* [68] has shown that isoform-selective activation of protein kinase C-epsilon (PKC_ε) is responsible for NO-mediated development of late preconditioning. To that end, they found that L-NA, a NO synthase (NOS) inhibitor, blocks the cardioprotective effect associated with late preconditioning with the simultaneous reduction in PKC_ε translocation. Furthermore, when preconditioning was mimicked by two structurally distinct NO donors, chelerythrine, a PKC_ε inhibitor, blocked the NO donor-mediated cardioprotection. This suggests perhaps that in delayed ischemic preconditioning, PKC_ε is the downstream effector of NO. Hence, under various cardiac pathologies, it is possible that protective effects attributed to AT_2R might involve NO-mediated PKC_ε activation to some extent. However, current support of this pathway is lacking.

Thus, delineation of AT_2R-mediated events in various pathophysiological states might give researchers a new tool to develop strategies against various cardiovascular diseases. Especially elucidation of these events will be beneficial in ischemia-reperfusion where activation or increase in factors such as kinins [102], NO [103-105], cGMP [106] and PKC_ε [68] have been implicated in acute cardioprotective effects.

Ischemia-Reperfusion Injury

Prompt reopening of the occluded vessel, either by thrombolytic therapy or angioplasty, prevents progression of cell necrosis in ischemic myocardium, a process often referred as

reperfusion. It has been clearly shown that although timely reperfusion is the most effective means of preventing ischemic damage, reperfusion may not be entirely beneficial. In this regard, studies have shown that untimely reperfusion triggers pathophysiological pathways that counteract some beneficial effects of the restoration of coronary flow [107]. These deleterious events during ischemia and reperfusion, collectively characterize ischemia-reperfusion (IR) injury.

Two of the major determinants of IR injury are the duration of myocardial ischemia and degree of perfusion in the ischemic myocardium. Limited duration of myocardial ischemia (less than 20 min) followed by reperfusion is accompanied by complete or partial functional recovery without structural or biochemical evidence of tissue injury [108]. In this instance, the myocardium may be characterized functionally by depressed myocardial contractility (myocardial stunning); however, recovery from this type of damage can be complete. Paradoxically, reperfusion of ischemic myocardium after extended periods of ischemia (> 20 min) can lead to an extension of the injury beyond that due to the ischemic insult itself. As such, instituting reperfusion, may trigger irreversible myocardial injury, characterized by cellular damage and cell death [108] via necrosis and apoptosis [109]. Since these events have varied clinical implications, both in pathological (myocardial stunning and infarction) and surgical settings (percutaneous transluminal coronary angioplasty (PTCA), cardiac surgery, cardiac transplantation etc) (Table 2), understanding the underlying pathophysiological events in IR are essential [110].

Over the years, many potential pathways leading to IR injury have been determined. However, the sequence of biochemical events whereby transient myocardial ischemia leads to functional and structural damage remains to be elucidated. Some of the

underlying events of IR injury includes (1) coronary endothelial dysfunction; (2) adherence of neutrophils to the endothelium, transendothelial migration, and the release of mediators; (3) acute diastolic dysfunction; (4) transient impairment of left ventricular systolic contractile function or myocardial stunning and; (5) arrhythmias [101] (Figure 4). Of these events, myocardial stunning perhaps received the most attention both in experimental and clinical settings.

Myocardial Stunning

Myocardial stunning was a term coined in 1982, and is defined as "the mechanical dysfunction that persists after reperfusion despite the absence of irreversible damage and despite restoration of normal or near-normal coronary flow" [111-113]. It occurs when a brief period of ischemia followed by reperfusion resulted in postischemic dysfunction [114]. Initially, the phenomenon was regarded largely as a laboratory curiosity. Subsequently, it gained attention among both experimentalists and clinicians. This was primarily because of two reasons. First, coronary reperfusion, by means of either thrombolytic therapy, PTCA or coronary artery bypass graft (CABG) surgery, has become a standard procedure for acute ischemic syndromes in patients with coronary artery disease. Second, several studies have demonstrated that many patients experience spontaneous reperfusion because of lysis of coronary thrombi. Thus, it has become increasingly evident that postischemic myocardial stunning is an essential part of the diagnosis of various diseases and post-operative assessment and is associated with significant morbidity and mortality in the patient population [107].

To date, a number of factors such as oxygen radicals, sarcoplasmic reticulum dysfunction, Ca^{2+} overload and reduced Ca^{2+} sensitivity have been implicated in the pathogenesis of myocardial stunning. The complex interplay between these pathways and its consequences is illustrated in Figure 4. Generation of oxygen-derived free radicals and disturbances in Ca^{2+} homeostasis are responsible for the postischemic contractile dysfunction [107]. These events are primarily triggered by activated neutrophils, impaired metabolism, catecholamine release and release of other vasoactive factors such as endothelins and metabolites of arachidonic acid [120]. Recently, Ang II has also been added to the list of these potential mediators [102, 119, 121-123].

Current experimental [124] and clinical studies [125] have hypothesized that acute myocardial ischemia is accompanied by activation of the RAS. This activation results in the formation of Ang II, which has deleterious consequences on ischemic tissue and ultimately contributes to the injury associated with acute myocardial infarction (MI). Recent studies have shown that during acute ischemia, the level of Ang II is increased in cardiac tissue, hence providing some evidence in support of prior speculations [126].

Potential Mechanisms of Ang II Cardiotoxicity

Ang II may activate several pathways in IR injury. Being a potent vasoconstrictor [124], Ang II can exacerbate ischemia-induced injury by decreasing coronary flow. Furthermore, Ang II increases sympathetic activation, which can result in cardiac arrhythmias because of the increase in norepinephrine [127]. Ang II receptors are coupled to phospholipase C, which stimulates DAG production and PKC activation, resulting in increased intracellular Ca^{2+} levels. During IR, such an increase in Ca^{2+} is detrimental, as

elevated Ca^{2+} levels activate various enzymes and kinases, which collectively contribute to the impairment of postischemic recovery [128]. Recently, Ang II has been implicated in neutrophil recruitment, which by virtue of their activation, releases various proinflammatory substances that contribute to IR injury [179, 180].

Ang II Therapies against IR Injury

Initially, because they were already being used for hypertension therapy, ACEI were also used to treat IR injury. Several lines of evidence suggest that the therapeutic effect of ACEI in IR is primarily due to blood vessel vasodilation, blunting of sympathetic activation and inhibition of bradykinin breakdown [125]. The inhibition of bradykinin breakdown is a major contributor to salutary responses of ACEI because bradykinin has been implicated in increasing NO and prostacyclin levels, and in reducing platelet aggregation, events that are cardioprotective [125, 129]. However, these initial studies with ACEI met with disappointments, as it was later discovered that Ang II could still be synthesized via alternative pathways [130]. Furthermore, side-effects such as a persistent annoying cough and angioneurotic edema became associated with the use of ACEI, which led to reduced patient compliance with the prescribed drug regimen [2]. These problems led to the development of AT_1R antagonists. This was especially helpful in light of current evidence, which suggests that neutrophils initially recruited by Ang II have the tendency to synthesize local Ang II, thereby augmenting the local Ang II pool significantly during IR [131].

The successful development of AT_1R antagonists in 1991 has provided researchers with a powerful tool to investigate the direct effects of Ang II in IR,

independent of its formation route. Preliminary experimentation with AT_1R antagonists showed some promising results. The AT_1R blocker L-158,338 attenuates the development of acidosis during 20 min of ischemia in isolated rat hearts, signifying that in experimental studies AT_1R can modulate ischemia-related metabolic alterations [115]. Furthermore, the AT_1R antagonist, losartan, decreases the incidence of ischemia-induced arrhythmia and attenuates the prolongation of transmural conduction time during 15 min of global ischemia in isolated guinea pig hearts. During the subsequent reperfusion, the duration of ventricular tachycardia and the incidence of ventricular premature beats were also decreased [116] with AT_1R blockade.

However, subsequent studies in isolated-perfused rat hearts with AT_1R antagonists have ignited controversy. Pretreatment with the AT_1R blockers, L-158,338 and losartan, increases coronary flow and cardiac output after 20 min of global ischemia in isolated working rat hearts [117]. When added to the perfusate in isolated rat hearts, losartan also increases coronary flow, the maximum of the first derivative of left ventricular pressure (LV dp/dt_{max}), and peak systolic pressure following 60 min of global ischemia [118]. In contrast, our laboratory showed that losartan in an isolated working rat heart model, when added 5 min prior to ischemia, worsens the recovery of left ventricular work after 30 min of global ischemia [132, 133]. In another study, losartan did not improve functional recovery of isolated guinea pig hearts after 15 min of global ischemia [119]. Apart from the different concentrations, the time of application and the model used, no obvious explanation exists for these apparent discrepancies in results, indicating the need for further studies.

Adding to the controversy, when myocardial infarct size was used as one of the end-points in various animal models after IR, losartan (a surmountable antagonist) showed no significant reduction in infarct size when compared to control [134]. This finding was further extended by various authors who found no change in infarct size under different IR protocols in different species [135-137]. On the other hand, candesartan (CAN, an insurmountable antagonist), showed a cardioprotective effect in various pathological conditions such as IR [121, 138] and myocardial infarction [100]. This beneficial effect might be attributed to different pharmacodynamic properties of the drugs, as they might differentially affect the recovery of the heart from pathological insults [138]. Despite the preliminary results of cardioprotection, the salutary effects of AT_1R blockade are not fully established.

Studies on the effects of AT_1R blockade in IR and analysis of other end-points such as infarct size, have further fueled to the controversy related to the effects of AT_1R blockade. In addition, recent evidence of different effects caused by AT_1R blockers with different pharmacodynamic properties further obscures our understanding of the precise effects of AT_1R blockade during IR [138]. Furthermore, recent concern over the increased plasma Ang II levels during AT_1R blockade and consequent end-organ damage, have further questioned the use of AT_1R blockers in IR [1]. Because of the negative effects of ACEI and AT_1R blockers, a low dose of combination of the two types of drugs as a potential therapy has been explored [1].

Combination Therapy of ACEI and AT_1R Antagonism

Use of ACEI and AT_1R blockade provides an attractive concept for therapy as the use of low doses of both drugs might additively increase the protective effect of therapies while reducing side-effects associated with larger dosages of individual treatments. Recent *in vivo* animal studies compared the effects of combined AT_1R antagonists and ACEI (over a wide range of doses) with their individual counterparts [139]. Combination therapy proved beneficial over a wide range of functional and morphological recoveries. Specifically, reductions in blood pressure, left ventricular body weight, cardiac hypertrophy and Ang II levels were observed in SHR. Unfortunately, the negative effects still persisted [139]. Hence, despite the recent strides made in elucidating the mechanisms of Ang II in various pathologies and development of therapies against Ang II, problems with the overactivation of this octapeptide still exists.

Purpose of the Study

Recent studies have elucidated a new mechanism by which augmentation of the therapeutic effect of AT_1R blocker can be achieved. Basic research has highlighted the importance of AT_2R. Studies in vasculature *in vitro* suggest that during AT_1R blockade, increased Ang II availability might stimulate AT_2R, triggering signal transduction pathways resulting in production of NO and cGMP [79-82, 85, 100, 101]. This increase was also systematically attenuated in the presence of AT_2R blockade. This suggests that activation of AT_2R causes increases in NO and [illegible]duction, which have been shown cardioprotective in nature [82, 100]. Hence, a co[illegible] an AT_1R blocker and an AT_2R agonist could be foreseen as a new therapeutic strategy against Ang II-mediated cardiac

pathologies. However, most of the studies, which have identified, isolated and characterized AT_2R were performed in cell culture and vascular tissues.

In this context, the goal was to examine the effects of AT_1R blockade and its AT_2R modulation in cardiac tissues, specifically, in the setting of IR. The specific objectives of this research project were:

1. To analyze if AT_2R content can be modulated by AT_1R blockade.
2. To determine if AT_1R blockade activates AT_2R.
3. To assess if any signal transduction pathways such as apoptosis, PKC_ϵ translocation and cGMP production are activated by AT_2R.

To achieve these objectives, experiments were carried out in two different isolated rat heart models. The first set of studies was performed in Langendorff perfused rat hearts. The underlying principle in this system is to perfuse the coronary vasculature with an oxygenated fluid (for our purposes at 70 mmHg pressure) through a cannula inserted in the aorta. In doing so, the coronary arteries are perfused, thus enabling the heart to work against the balloon inserted in the ventricle. This balloon is in turn attached to a pressure transducer, which records myocardial functional parameters. Another parameter, coronary flow, was determined by the flow rate of the effluent from the heart chamber. The reason for choosing this approach was because numerous studies published in the literature [102, 120-123] examined the effects of AT_1R blockade using this model and found a cardioprotective effect. Since previous studies published from this laboratory showed no such effect of AT_1R blockade in isolated working rat heart [132, 133], Langendorff perfused hearts were chosen to determine whether the difference in results with the AT_1R antagonist might be due differences in the model.

After the first set of studies was completed, the second set of studies was transferred to the isolated working rat heart model. The isolated working rat heart is closer to the *in vivo* setting. In this model, the hearts are perfused at a physiological preload and afterload. The work output of working rat hearts is similar to *in vivo* values and considerably higher than in the non-working (Langendorff) heart [140]. As the name of the model suggests, the heart works or pumps the perfusate against the preset afterload. In doing so, the hearts i) perform external work by ejecting against the set hydrostatic pressure, or afterload, ii) perfuse their own coronary circulation, and iii) generates ATP from the oxidation of fatty acid (palmitate) present in the perfusate (which is unique to this setting). The presence of fatty acids in the perfusate of working hearts, in addition to glucose and insulin, is critical because i) high fatty acid levels are clinically relevant in most acute coronary syndromes and ii) fatty acid oxidation is the major source of ATP during aerobic metabolism and during postischemic reperfusion [141, 142]. Thus, the isolated working rat heart reflects the setting quite close to *in vivo* conditions found in patients.

Cardioprotection was used as a primary end-point for both sets of experiments. Classically, cardioprotection has been defined as "all mechanisms and means that contribute to the preservation of the heart by reducing or even preventing myocardial damage" [143]. This damage can be manifest by increased dysrhythmias, increased infarct size and/or decreased post-ischemic recovery. Since experiments described in this thesis involve a myocardial stunning model (no dysrhythmia was detected during the course of experimentation), post-ischemic recovery of mechanical contractile function was the primary end-point. Therefore, cardioprotection, for our purposes, was defined as the extent of the post-ischemic recovery observed in hearts subjected to IR, as assessed by

left ventricular developed pressure (LVDP) in Langendorff-perfused rat hearts and LV work in isolated working rat hearts. Hence, these indices were the primary end-points used to assess the effectiveness of AT_1R antagonists on the functional recovery of rat hearts subjected to IR.

Moreover, using these two models, the effects of AT_1R blockade in relation to AT_2R protein, and apoptosis were assessed in Langendorff perfused hearts. Studies were extended to the analysis of AT_2R activation and its consequent effects on various signal transduction molecules (cGMP, PKC_ε) in the isolated working rat heart preparation.

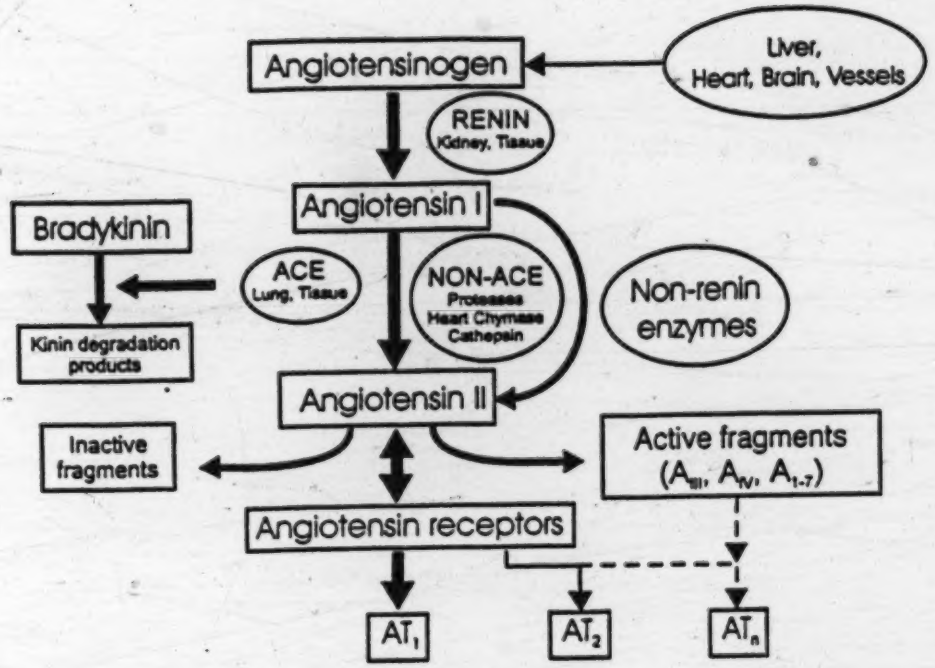

Figure 1 Current view of the angiotensin system. A= angiotensin; ACE= angiotensin-converting enzyme; AT= angiotensin subtype receptor. Reproduced with permission. Reproduced from Jugdutt [1]

Table 1. SOME ACTIONS OF ANGIOTENSIN II

Target	Circulating Renin-Angiotensin System	Tissue Renin-Angiotensin System
Heart	Positive inotrope, contractility	Cardiac remodeling
	Chronotrope	Myocardial hypertrophy
	Enhance sympathetic activity	Fibroblast proliferation
	Coronary constriction	Fibrosis
	Ventricular arrhythmias	Increased diastolic stiffness
	Myocardial metabolism	Apoptosis
Vessels	Direct vasoconstriction	Vascular remodeling
		Vascular hypertrophy
		Smooth muscle proliferation
		Apoptosis
		Coagulation and fibrinolysis
		Superoxide production
Brain	Vasopressin release	
	Stimulate thirst	
	Catecholamine release	
	Increase noradrenaline	
Sympathetic nervous system	Increase central sympathetic outflow	
	Increase noradrenergic activity	
	Increase adrenal adrenaline release	
	Facilitate peripheral sympathetic activity	
Kidney	Sodium and water retention	Intraglomerular hypertension
	Increase sodium resorption	Hypertrophy
	Increase blood volume	Embryogenesis
	Inhibit renin release	
	Release prostaglandins	
Adrenal	Aldosterone secretion	

Reproduced from Jugdutt [1]

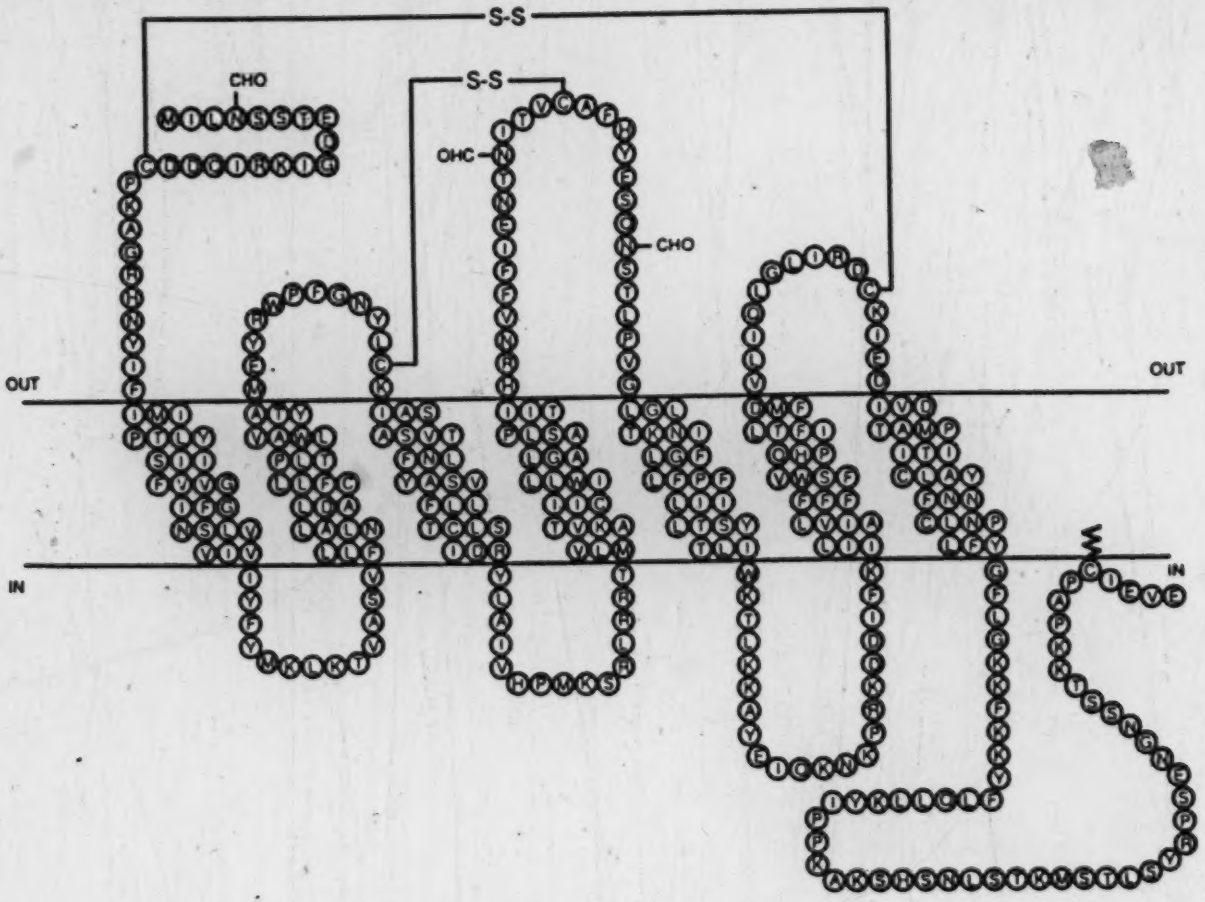

Figure 2 Putative two dimentional organization of bovine AT_1R. Reproduced from Inagami *et al* [32]

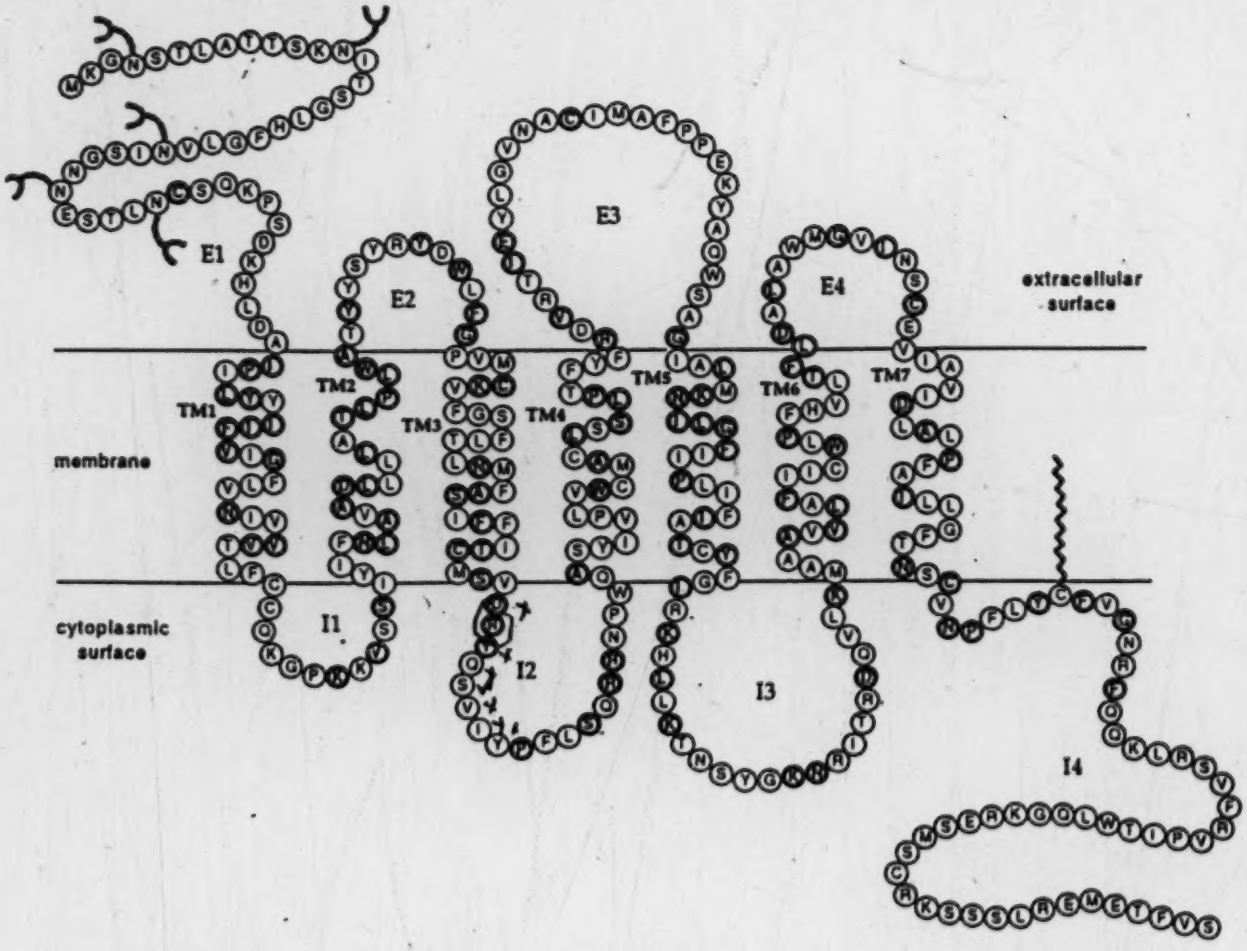

Figure 3 Putative two dimentional organization of bovine AT_2R. Reproduced from Nahimas *et al* [181]

Table 2. Clinical settings which are associated with ischemia-reperfusion and their experimental equivalents.

Experimental Setting	Clinical Setting
Regional ischemia	
Completely reversible ischemic episode (coronary occlusion < 20 min)	PTCA Unstable angina Variant angina
Partly irrevesible ischemic episode (subendocardial infarction, coronary occlusion > 20 min, < 2 hours)	Acute myocardial infarction with early reperfusion
Exercise-induced ischemia in presence of coronary stenosis	Exercise-induced ischemia in presence of coronary stenosis
Global Ischemia	Cardiac surgery
Cardioplegic arrest	Cardiac transplantation Cardiac arrest
Exercise-induced ischemia in hypertrophic hearts	Exercise-induced ischemia in hypertrophic hearts

Modified and adapted from Bolli *et al* [170]

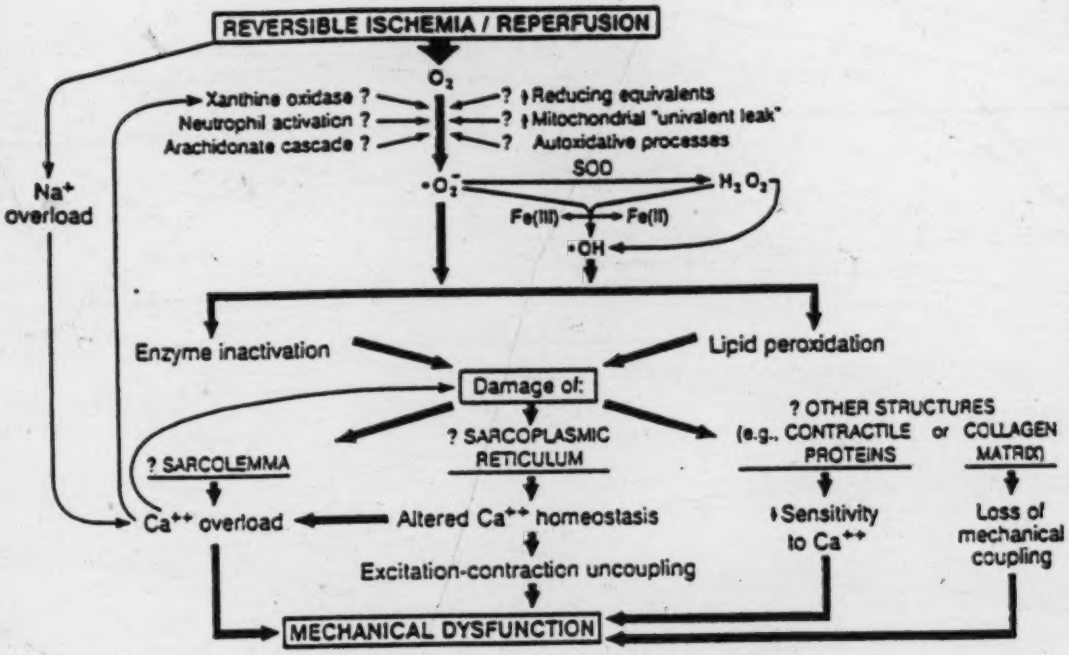

Figure 4 Possible pathophysiological mechanisms involved in myocardial stunning. Reproduced from Bolli *et al* [110]

METHODS

Animals

All experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care and the American Physiological Society and were approved locally by the Health Sciences Laboratory Animal Welfare Committee of the University of Alberta. After acclimatization for 1 week, pathogen-free male Sprague-Dawley rats were randomly assigned to various groups according to the perfusion protocols. The rats were weighed and anesthetized with sodium pentobarbital (120 mg/kg, i.p.). Thereafter, a standardized surgical procedure, which results in a very low rate of technical failure and increases stability of heart preparations, was used [132, 133, 144]. Briefly, after induction of anesthesia, as judged by loss of pedal and palpebral reflex responses, the abdomen was opened and an incision was made in the chest exposing the thoracic cavity and the heart. Subsequently, the hearts were excised and mounted on perfusion apparatus for either Langendorff or working mode perfusion.

Preparation of Hearts for Langendorff Perfusion

In this set-up, hearts were perfused under a constant-pressure (70 mmHg), non-recirculating Langendorff mode, using a modified Krebs-Henseleit (KH) solution that contained, in mM: NaCl 118, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.66, $CaCl_2$ 1.5 and glucose 10. This solution was filtered through 0.45 µm nitrocellulose filters and then gassed continuously with a mixture of 95% O_2 / 5% CO_2. This resulted in a solution with a pH of 7.4 to 7.45, and a free ionized Ca^{2+} of 1.25 mM, as previously described [144]. During

the experiments, the temperature of the perfusate was continuously monitored by a scanning thermocouple analyzer (model 692-8010, Barnant Co., Barrington, IL, USA) and the perfusate reservoir and the solution in the heart chamber were maintained at 37.7° ± 0.1°C and 36.7° ± 0.1°C, respectively.

After each heart was mounted on the perfusion apparatus, extraneous tissue such as lungs, thymus and atria were excised. A polyethylene balloon [145] (volume 0.52-0.90 cm^3), that was filled with degassed distilled water, was inserted into the left ventricle (LV) through the mitral valve. The sino-atrial node was mechanically obliterated and the heart was artificially paced at 5 Hz (Grass S88 stimulator, Grass Instruments Co., Quincey, MA, USA) throughout the experiment.

Assessment of Mechanical Function in Langendorff-perfused Hearts

For monitoring the indices of cardiac mechanical function, LV end-diastolic pressure (LVEDP) was measured by a transducer connected to the polyethylene balloon present in the LV. LVEDP was adjusted to 4-6 mmHg at the beginning of each experiment and the volume of the balloon was kept constant throughout the remainder of the experiment. Functional parameters (LV systolic pressure (LVSP, mmHg), LV developed pressure (LVDP, mmHg), heart rate (HR, bpm) and LVEDP (mmHg) were recorded via a computer-based system (Digi-Med™ Heart Performance Analyzer, Micro-Med Inc., Lousville, KY, USA). Coronary flow (CF) rate was monitored throughout the experiments by 2N transonic flow probes attached to a T206 transonic flow meter (Transonic Systems Inc., Ithaca, NY, USA) which in turn was connected to the Digi-Med™ analog signal analyzer via the voltage output from the transonic system.

Perfusion Protocols

A. Preparation of CAN solutions

Initial experiments with candesartan (CAN) showed the need of resetting stock pH. It was found that resetting CAN solution from 8-8.5 to pH 7.4 (n=3) resulted in an improved postischemic LVDP recovery when compared to non-resetted pH of CAN solution (n=3) and the matched controls (n=7) (Figure 5; $p<0.05$). Therefore, subsequent experiments were carried out using CAN that was dissolved in 0.9% physiological saline, in combination with sodium carbonate (adding 100 µl (1 M) in 2 ml of stock solution), with resetting of pH to 7.4.

B. Concentration-Response Relationship for CAN

To determine the effective concentration of CAN, the ability of increasing concentrations of CAN (1, 10, 100 nM) to reverse Ang II-induced coronary vasoconstriction was assessed. After an equilibration period of 30 min, administration of Ang II (0.1 µM) over 3 min elicited a 30% decrease in coronary flow. Subsequent perfusion with CAN (1, 10, 100 nM, n=4 for each group) for 40 min revealed that 1 nM of CAN had a marginal effect on the Ang II-induced decrease in coronary flow, while 10 nM and 100 nM of CAN similarly abrogated the response, suggesting that a plateau of inhibiton was reached with 10 nM of CAN. We therefore used 10 nM of CAN, as done by other investigators [113,124] in our subsequent experiments.

C. Duration of Exposure to CAN

Initial studies done to examine the effects of CAN on recovery of postischemic function used a protocol, where CAN was administered 5 min before ischemia and in reperfusion. With this treatment protocol, there was no significant difference in the recovery of LVDP

between CAN and matched controls (29±10; n=6 versus 26±5 mmHg; n=13), although later analysis with TUNEL assay showed a significant reduction in apoptosis in CAN treated hearts (Figure 6; $p<0.05$). Subsequent review of the literature suggested that CAN might be more effective when present for at least 30 min before the ischemic insult. Thus, a new protocol, in which CAN was present for 40 min prior to the onset of ischemia, was evaluated.

D. IR and Aerobic Protocols

Under this protocol, 'untreated hearts' were subjected to 50 min of baseline aerobic perfusion followed by 30 min of global no-flow ischemia and 40 min of aerobic reperfusion (n=6). In the treated group, CAN was added after 10 min of baseline perfusion and remained throughout the remaining 40 min of baseline aerobic perfusion. CAN was also present during no-flow global ischemia, however, no drug was present during reperfusion. At the end of reperfusion in both groups, hearts were sliced and preserved in formalin and liquid nitrogen for apoptotic and molecular studies, respectively. Additional groups of hearts were preserved for analysis of AT_1R/AT_2R proteins and apoptosis in the presence or absence of CAN, after i) perfusion for 50 min (Pre-I, n=3), ii) perfusion for 50 min followed by 30 min of ischemia (End-I, n=3), or iii) aerobic perfusion for 2 h (Aerobic, n=3) (Figure 7). These additional hearts were perfused to determine if differences observed in biochemical indices between control and CAN groups were due to CAN *per se* or the presence of CAN during specific condition periods within the perfusion protocol.

Preparation of Hearts for Working Mode Perfusion

The aorta was cannulated and Langendorff perfusion was initiated and performed for 10 min at a hydrostatic pressure of 60 mmHg with a modified KH solution of the following composition (in mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 3.0, EDTA 0.5 and glucose 11 at pH 7.4. The solution was in contact with 95% of O_2 / 5% CO_2. During the initial Langendorff perfusion, extraneous tissue such as lungs and thymus were excised and the left atrium was cannulated with subsequent switching of the heart from Langendorff (by clamping off aortic inflow line) to working mode (by unclamping left atrial inflow and aortic outflow lines) using Neely's method [146]. In working mode, hearts were perfused under aerobic conditions with modified KH solution containing, in addition to above constituents 0.5 mM of lactate, 100 μU/mL of insulin and 1.2 mM of palmitate pre-bound to 3% bovine serum albumin. This perfusion was carried out in a closed re-circulating system at 37° C in close contact with the 95% O_2 / 5% CO_2 mixture. The solution entered the left atrium at a preload of 11.5 mmHg and ejected against a column height of 80 mmHg. Atrial pacing was applied at 5 Hz (Grass S88 stimulator) after 5 min of initial aerobic working perfusion and after 5 min of aerobic reperfusion. Hearts were not paced during ischemia.

Assessment of Mechanical Function in Isolated Working Rat Hearts

Heart rate and systolic and diastolic pressures were recorded (Gould 2800S model polygraph, Cleveland, OH, USA). Collection of data from working hearts was done by a computer-based system (Digi-Med™ Blood Pressure Analyzer, Micro-Med Inc., Lousville, KY, USA). Cardiac output and aortic flow were measured by 2N transonic flow probes

attached to a T206 transonic meter which in turn was attached to the Digi-Med™ analog signal analyzer. These probes were placed in left atrial inflow (cardiac output) and aortic outflow (aortic flow) lines, respectively. Coronary flow (ml/min) was calculated as the difference between cardiac output and aortic flow. LV work (Joules) was calculated as the difference between systolic pressure and preload pressure times the cardiac output times 0.133.

Working Heart Perfusion Protocols

We studied the effects of pretreatment of CAN (10 nM) on recovery of LV mechanical function after IR (25 min ischemia and 40 min reperfusion). Effects of another AT_1R antagonist (losartan; 1μM), an AT_2R antagonist (PD 123,319; PD; 0.3 μM), and their combination (PD+CAN) were assessed under the same protocol. Each drug was given before, during and after ischemia (Figure 8). Furthermore, aerobic protocols were also performed for each group (Figure 9) involving a 115 min of aerobic perfusion. The drugs were administered 10 min into baseline perfusion and were present for the remainder of 105 min of aerobic perfusion.

Assessment of Myocardial Protein Content by Immunoblot Analysis

A. Content of AT_1R and AT_2R

Frozen LV tissue samples (10 mg) were pulverized, weighed and homogenized in 0.15 ml of homogenising buffer. The homogenates were sonicated at 4°C in homogenizing buffer (2% SDS sodium dodecyl sulphate (SDS), 100 mM dithiothreitol, 60 nM Tris, pH

6.8). The samples were tested for amount of protein present by the Bradford assay and were subsequently normalized. After normalization, the samples were treated with blue dye and were boiled at 100 °C. Samples were loaded in different wells of 9% SDS polyacrylamide gel with molecular markers on either side for comparison of molecular weights. The gel was subjected to electrophoresis, which was followed by electrotransfer to nitrocellulose membranes using a Mini-Trans blot electrophoretic transfer system (Bio-Rad, Hercules, CA, USA) at 4°C. Non-specific binding of proteins on nitrocellulose membranes was then blocked with phosphate-buffered saline (PBS) supplemented with 5% (wt/vol) of skimmed milk and 0.05% (vol/vol) of Tween 20 at room temperature.

Quantification of AT_1R and AT_2R Protein Contents

The membranes were incubated with affinity-purified rabbit anti-human AT_1R antibody at a dilution of 1:2000 for 2 hr at room temperature. The membranes were then washed 3 times with PBS-Tween 20 (0.05%, TPBS), incubated with goat anti-rabbit IgG antibody and visualized using chemiluminescence detection. The intensities of the bands were quantified by scanning densitometry using standard image analysis software (Sigma Gel, SPSS Inc., Chicago, IL, USA) and images were aligned with intensity bars for illustrations. For AT_2R protein content, the same procedure as for AT_1 was used except that incubations were performed with goat anti-human AT_2 antibody at a dilution of 1:500 and this was followed by incubation with donkey anti-goat IgG at a dilution of 1:4000.

B. Content of PKC_ε Activity

Frozen LV tissue samples were powdered and weighed (~ 100 mg). Tissues were homogenized in small glass on glass homogenizers in solution (0.10 ml) containing 150 mM NaCl, 40 mM NaF, 50 mM Tris pH 7.2, 1% Nondet P-40, EDTA 5 mM and EGTA 5 mM. The cytosolic and particulate portions of total cellular proteins were separated by centrifugation at 15,000 g for 60 min. The supernatant was collected and reserved as a cytosolic fraction. The resulting pellet was resuspended in high detergent solution containing 1% Na deoxycholate and 1% SDS in addition to the above-mentioned constituents [68]. Protein concentration was assessed by the Bradford protein assay (Pierce, Rockford, IL, USA). Western immunoblots were performed on both cytosolic and particulate fractions in a manner identical to AT_1R except that incubation with goat anti-human $nPKC_\varepsilon$ antibody at a dilution of 1:500 was followed by incubation with donkey anti-goat IgG at a dilution of 1:4000. PKC_ε activity was determined by ratio of the protein content in particulate to cytosolic fractions.

Determination of Myocardial cGMP Content

Frozen myocardial tissue (100 mg) was used for the determination of cGMP content. After pulverization and solubilization in 6% (v/v) trichloroacetic acid containing 10 mM HCl, a final concentration of 100 mg tissue in 1 ml solution was achieved. Centrifugation at 2000 g for 15 min was carried out and the resulting supernatant was washed with water-saturated diethylether to remove trichloroacetic acid. Subsequently, the cGMP-containing liquid phase was lyophilized and cGMP content in the ventricular extract was determined using a commercially available cGMP enzyme immunoassay kit. Protein was

measured by microplate Bradford assay. LV cGMP contents were expressed as fmol/mg protein [147].

Nuclear Morphology and TUNEL (Terminal Deoxynucleotidyl Transferase [Tdt] Mediated dUTP Nick End Labeling) Assay

Fresh hearts were sectioned into 5 mm transverse slices and fixed in neutral formalin (4% formaldehyde in 0.15 M phosphate buffer, pH 7.4). As described previously [144], up to 35 formalin-fixed sample hearts were embedded in paraffin, sectioned and analyzed simultaneously in a systematic fashion. Samples of rat jejunum and spleen were used as positive and negative controls, respectively. As reported by others [148], we confirmed that Tdt-positive cells were detected mostly at the tip of the villi of the intestinal epithelium. After dewaxing and rehydration, tissue sections (5 μm) were permeabilized with methanol/acetone (1:1) for 10 min at room temperature, washed twice with phosphate-buffered saline and incubated at 37°C with 50 mM proteinase K in 50 mM Tris/HCl (pH 7.5), 5 mM $CaCl_2$, for 15 min. Slides were incubated at room temperature with 2% H_2O_2 for 5 min to inactivate endogenous peroxidase enzyme.

DNA fragmentation was assessed by using the ApopTag® kit, which utilizes TdT for the addition of digoxigenin-dUTP to the 3′-OH ends of the DNA strand breaks [149] and direct immunoperoxidase detection of the digoxigenin-labeled 3′-OH ends. For every block, positive controls (by pretreating the tissue sections with 1U/mL DNAase I, for 15 min at 37° C) and negative controls (by omitting digoxigenin-dUTP or TdT) were run. Slides were then mounted, counterstained with chloroform-purified methyl green (i.e. no cytoplasmic counterstaining) and examined with a Nikon Labophot II (Nikon, Tokyo,

Japan). A minimum of 3,000 nuclei/heart were carefully examined at a magnification of x1250 after methyl green counterstaining. All slides were coded and codes were only broken after completion of microscopic analysis. Morphological analysis was used to classify nuclear changes in each TUNEL-positive apoptotic cell into 4 categories as follows [144, 149] 1) "early" apoptosis, with intact nuclei or nuclei with minimal chromatin condensation residing in intact myocytes; 2) condensed (pyknotic) nuclei without fragmentation and myocytes with condensed cytoplasm; 3) nuclear fragments and usually ruptured myocytes; 4) apoptotic bodies from ruptured myocytes (Figure 14). Scattered apoptotic bodies were not counted. TUNEL-positive nuclei with or without fragmentation in categories 2, 3 and 4 were considered to represent "late" apoptosis. Photographs were recorded (Zeiss microscope and Kodak Ektachrome reversible film) and scanned for prints (Digital Science™ Photo CD software, Eastman Kodak Co., Rochester, NY, USA). Apoptotic index was expressed as percent TUNEL-positive cells, which is based on the total number of apoptotic events detected per 1000 of nuclei counted.

Materials

Candesartan (CAN) (Astra Pharm Inc., Missisauga, ON, Canada) and losartan (Merck Frosst, Montreal, QB, Canada) were generous contributions from the respective companies. PD123319 was purchased from RBI (Oakville, ON, Canada). Purified rabbit anti-human AT_1R antibody, goat anti-human AT_2 antibody and goat anti-human $nPKC_\varepsilon$ were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA. Donkey anti-goat IgG was acquired from BioCan Scientific, Mississauga, ON, Canada. cGMP enzyme immunoassay kit was bought from Amersham Pharmacia Biotech UK Limited,

Buckinghamshire, England, UK. Chemiluminescence detection was acquired from ECL Amersham, Piscataway, NJ, USA. Chemicals for assessment of apoptosis were purchased from Sigma Chemical Co., St. Louis, MO, USA, and proteinase K and ApopTag® *in situ* apoptosis detection kit was obtained from Oncor, Inc. Gaithersburg, MD, USA.

Statistics

Data were analyzed using one way analysis of variance (ANOVA) followed by pairwise multiple comparisons (Student-Newman-Keuls Test) for the significance of differences between the groups. Repeated measures ANOVA was used for comparing serial data within groups. Values are shown as mean ± SEM. Differences were judged to be significant when $p<0.05$.

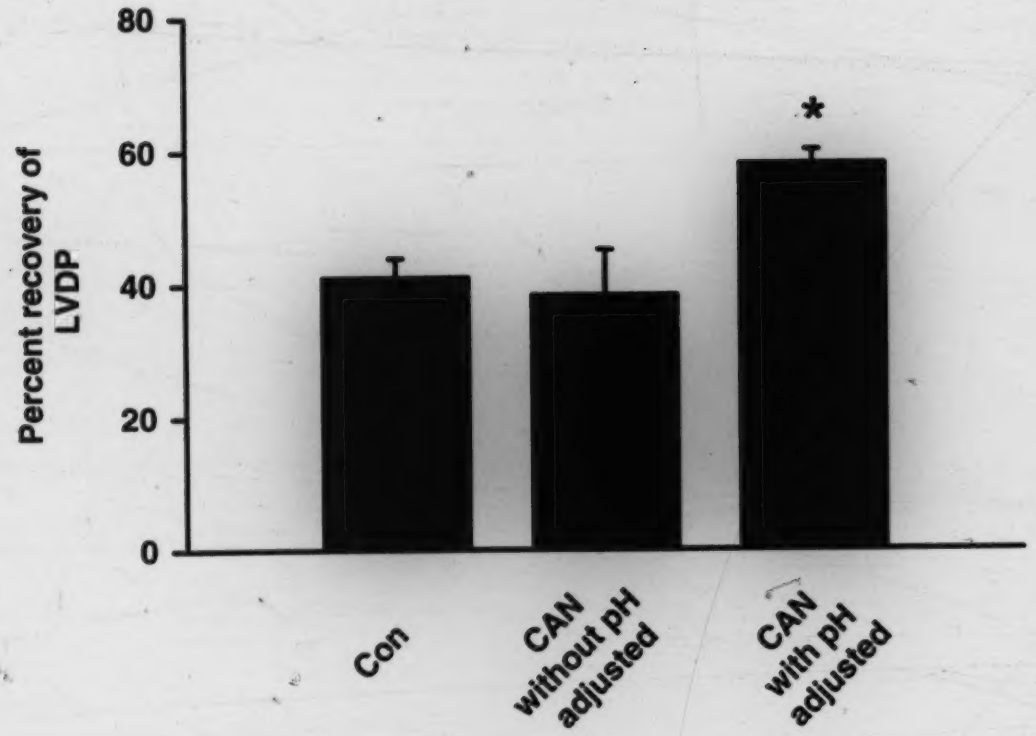

Figure 5 Effect of pH adjustment of candesartan (CAN) stock solution on percent recovery of LVDP. *p<0.05 when compared to controls (Con).

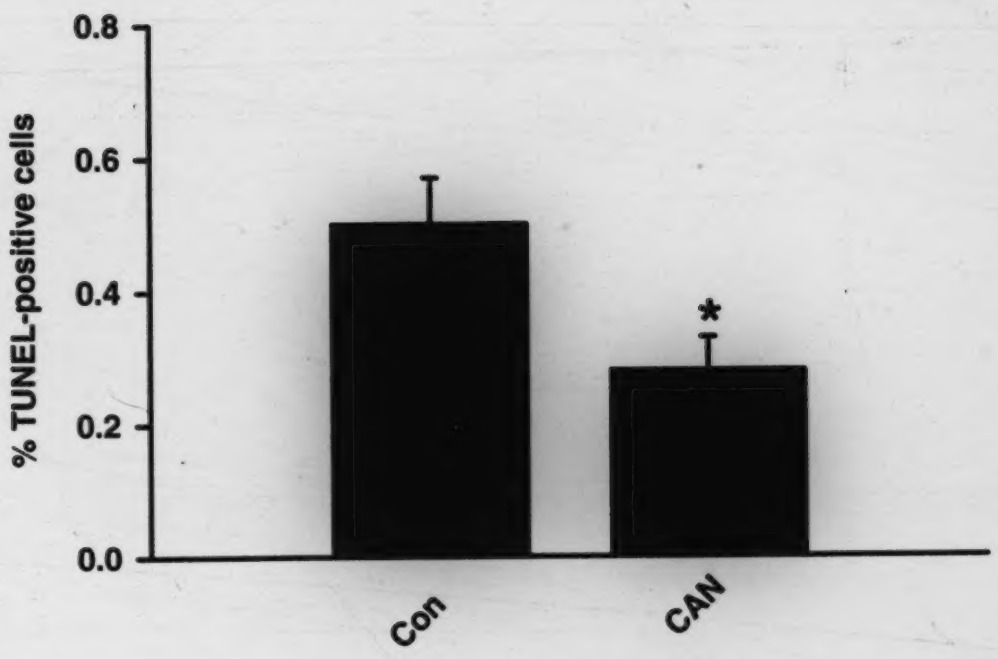

Figure 6 Effect of candesartan (CAN) on apoptosis in hearts after ischemia-reperfusion. Percent TUNEL positive cells= number of TUNEL positive cells among 1000 nuclei counted, expressed as percent. * $p<0.05$ when compared to controls (Con).

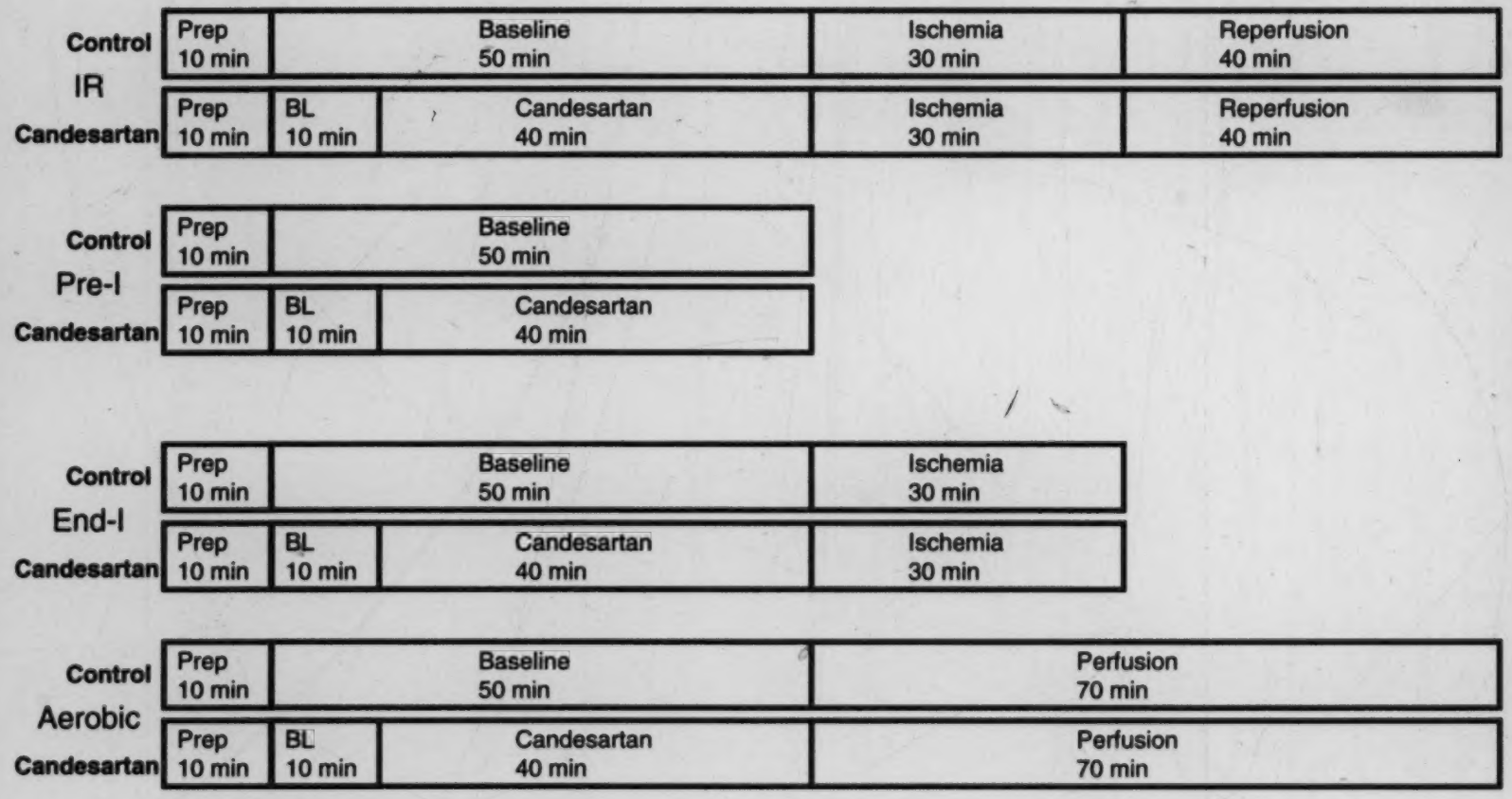

Figure 7 Schematic diagram of the 4 different groups and Langendorff perfusion protocols.

BL= Baseline perfusion; Prep= Preparation.

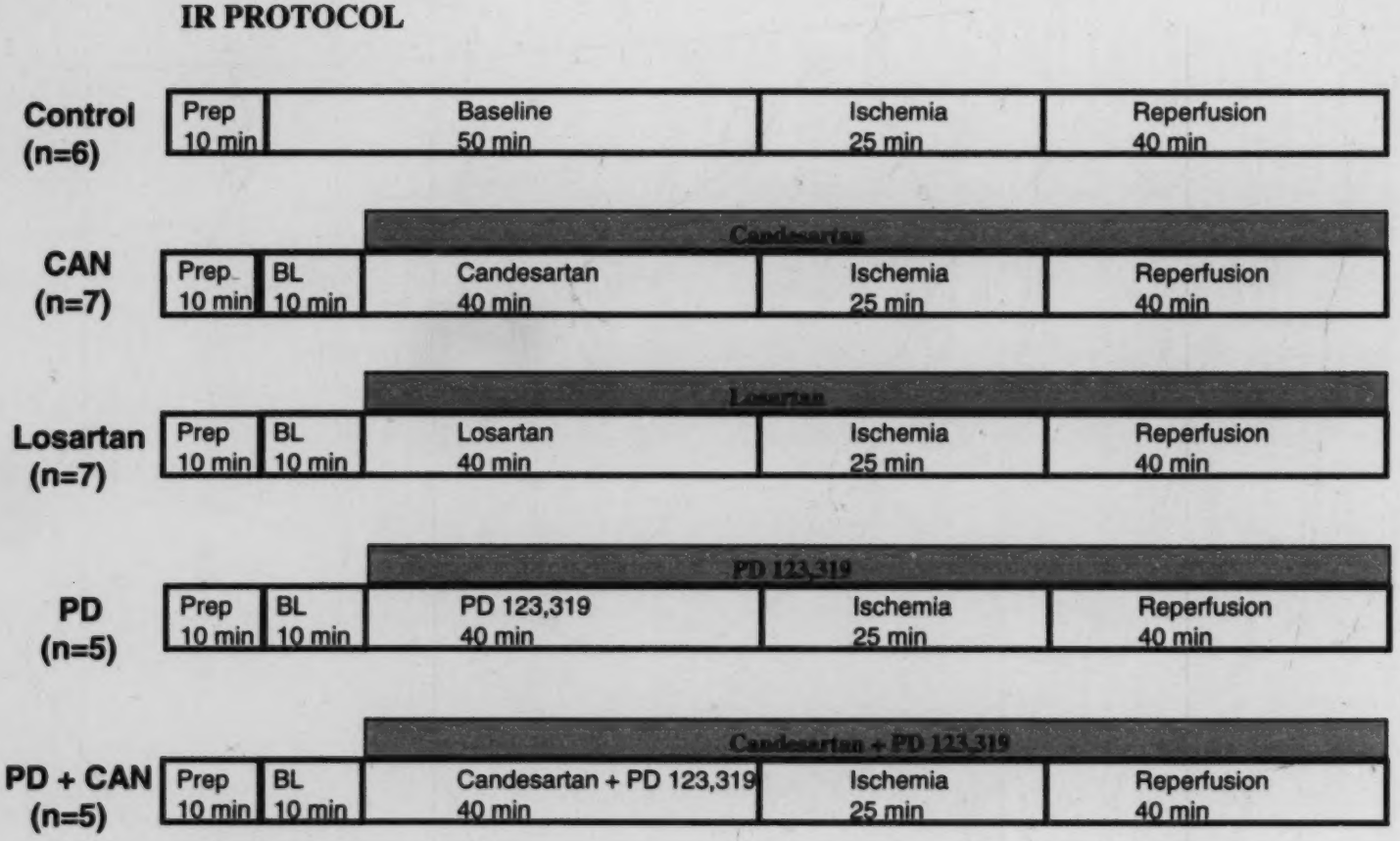

Figure 8 Schematic diagram of 5 different groups in IR protocol in isolated working rat heart. BL= Baseline Prep=Preparation

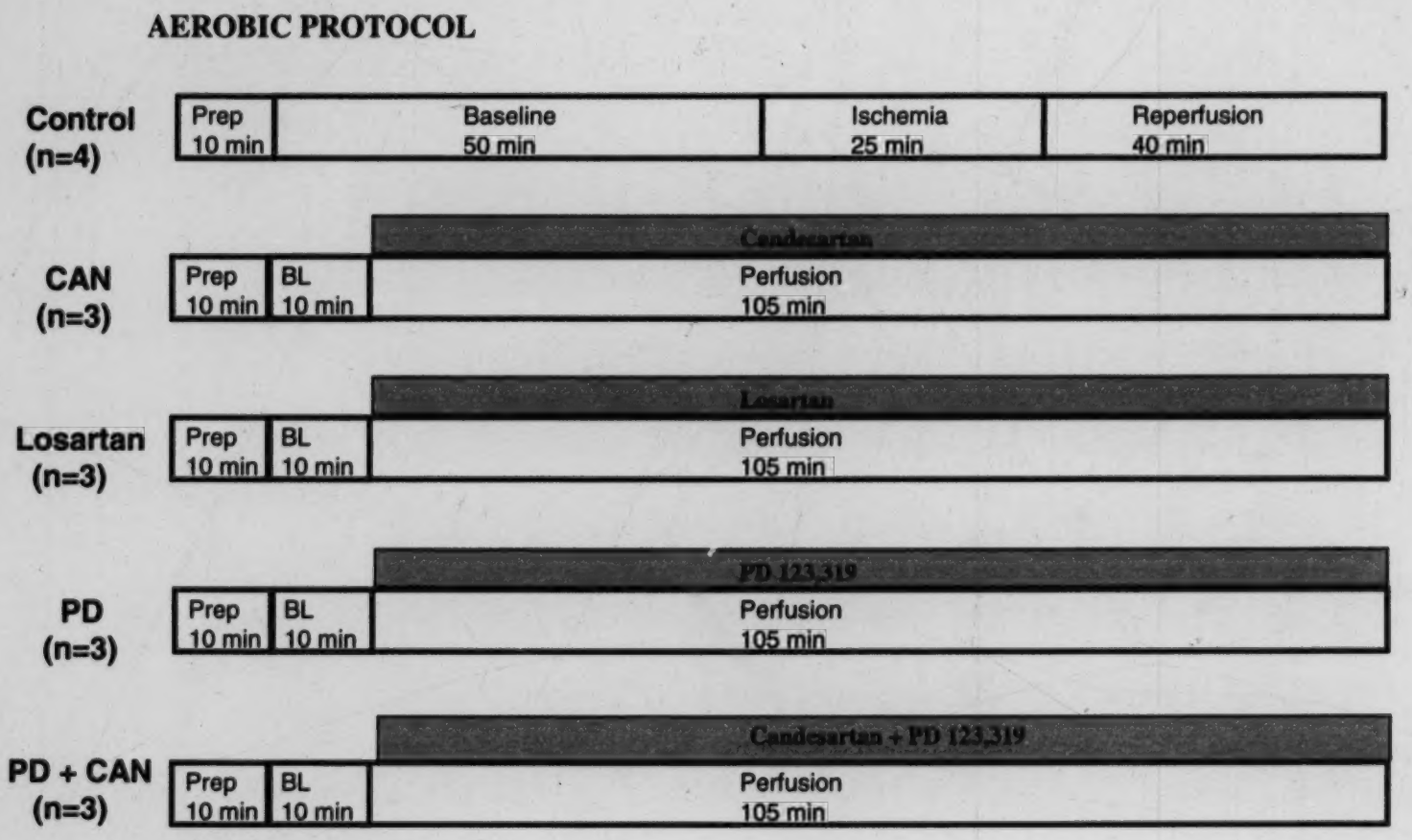

Figure 9 Schematic diagram of 5 different groups in aerobic protocol in isolated working rat heart. BL= Baseline Prep=Preparation

RESULTS

A. EXPERIMENTS USING LANGENDORFF PREPARATION

Effects of CAN on Mechanical Functional in Langendorff-perfused Hearts

LV mechanical function, as judged by LVDP, LV $+dp/dt_{max}$, and CF, were stable in untreated hearts during the first 50 min period of aerobic perfusion. When hearts were subjected to global no-flow ischemia, mechanical function rapidly ceased (<1 min). LVEDP increased gradually and, by the end of the 30 min period of ischemia, had increased to 72±3 mmHg. Upon reperfusion, mechanical function resumed slowly and by the end of reperfusion LVDP and LV $+dp/dt_{max}$ had recovered to 11.7±4.9% (Figure 10A) and 10.7±3.7% (Figure 10B), respectively, of pre-ischemic values. LVEDP declined slightly towards baseline values and after 40 min of reperfusion remained elevated (59±4 mmHg) relative to the pre-ischemic value (4±1 mmHg) (Table 3). During reperfusion CF recovered to 48.4±4.3% of the pre-ischemic value (Table 3).

CAN, when present only between 10 and 50 min of baseline aerobic perfusion had no effect on mechanical function or CF in any of the four perfusion groups. However, in the group of hearts that were subjected to IR, CAN pretreatment caused a significant increase in recovery of LVDP (43.3±7.9%; Figure 10A; $p<0.05$) and LV $+dp/dt_{max}$ (34.7±6.4%; Figure 10B; $p<0.05$) at the end of the reperfusion. This CAN-mediated increase in LVDP at the end of reperfusion was largely due to increase in LVSP (91±3 versus 70±4 mm Hg; Table 3; $p<0.05$) as no difference in LVEDP (54±4 mmHg)

was observed between groups at the end of reperfusion. Moreover, CAN pretreatment had no effect on recovery of CF (65.0±5.9%) at the end of reperfusion (Table 3).

In the group of hearts subjected to prolonged aerobic perfusion without IR, untreated hearts showed stable LVDP, LV $+dp/dt_{max}$ and CF for the first 80 min, thereafter a steady decline (~10%) in all of the functional parameters was observed during the remaining of 40 min in reperfusion. Hearts that were pretreated with CAN also showed a similar functional profile, that indicated that CAN pretreatment had no effect on any of the parameters of mechanical function in Aerobic perfusion.

Effects of CAN on AT_1R and AT_2R Protein Content in Langendorff-perfused Hearts

The protein contents of AT_1R, that were determined in tissue frozen at the end of each perfusion protocol (IR, Pre-I, End-I and aerobic groups) were similar in untreated and CAN pretreated groups (Figure 11A and 12A).

CAN significantly increased AT_2R protein content at the end of the IR protocol (Figure 12B; $p<0.05$). This CAN-mediated increase in AT_2R protein content seems to require ischemia as AT_2R was also increased in hearts frozen at the end of ischemia (End-I; Figure 11B; $p<0.05$). However, the protein contents of AT_2R that were determined in tissue frozen at the end of aerobic perfusion protocol (either Pre-I or prolonged aerobic perfusion) were similar in untreated and CAN pretreated groups (Figure 12B).

Effects of CAN on Apoptosis in Langendorff-perfused Hearts.

The incidence of apoptosis, as indicated by TUNEL-assay, were low in untreated hearts frozen prior to ischemia (Pre-I), at the end of ischemia (End-I), at the end of reperfusion (IR; Figure 11) and at the end of prolonged aerobic perfusion (Aerobic). The incidences of apoptosis were not affected by CAN-pretreatment. Detailed morphological analysis of untreated and CAN pretreated hearts further confirmed the lack of effect in the early (category 1) to late apoptosis (category 2,3 and 4) ratio (Table 4). Category 1, 2, 3, and 4 is illustrated in Figure 14.

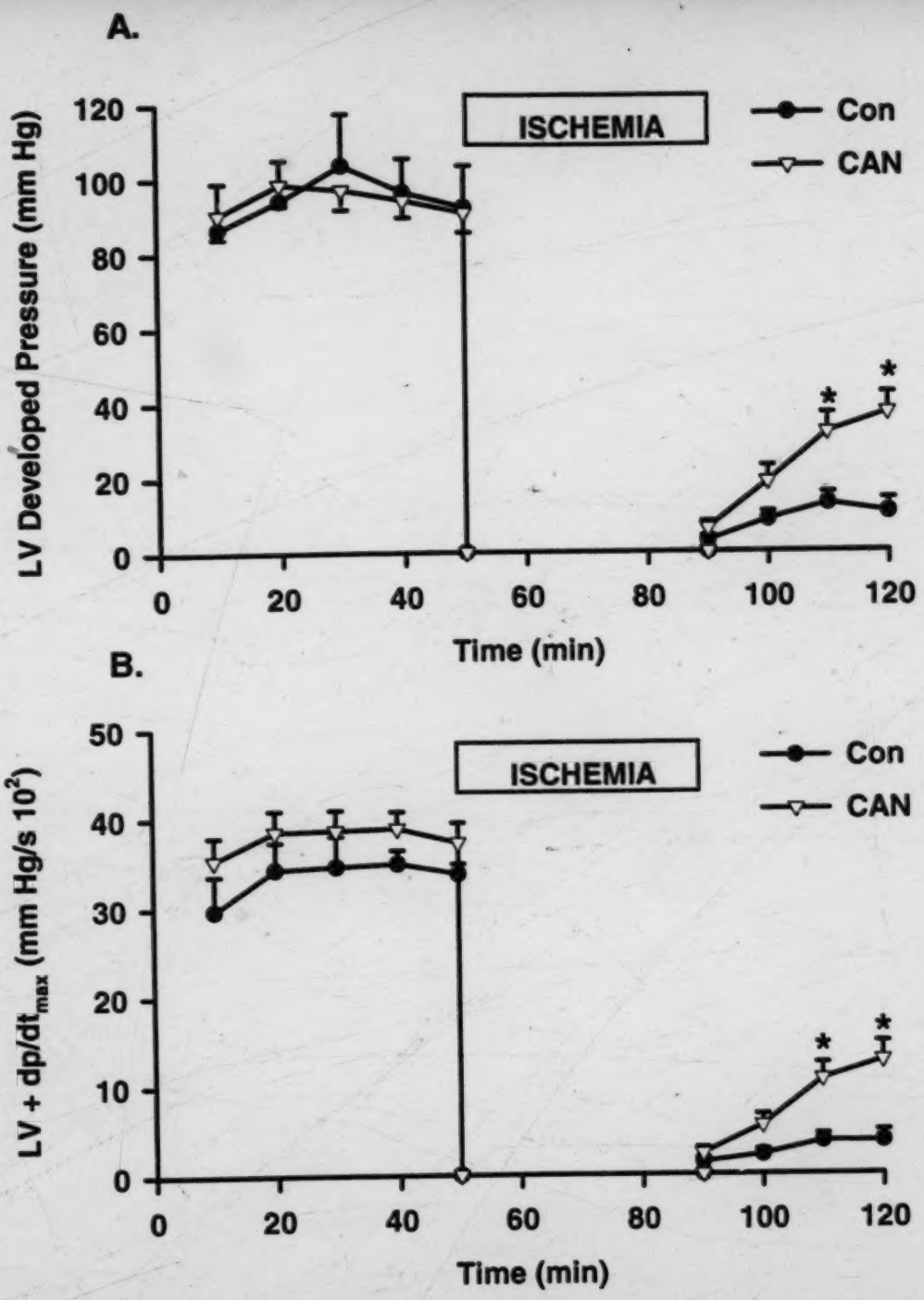

Figure 10 Effect of candesartan (CAN) on the recovery of left ventricular (LV) developed pressure (A) and LV $+dp/dt_{max}$ (B) after 30 min of global ischemia in Langendorff preparation. *p<0.01 to 0.001, comparing CAN to controls (Con) at corresponding timepoints.

Table 3. Effects of Pretreatment With Candesartan on Recovery of Function After 30 Minutes of Global Ischemia

Functional Parameters	Preischemia		Reperfusion	
	Control (n=6)	Candesartan (n=9)	Control (n=6)	Candesartan (n=9)
Coronary Flow (ml/min)	14±1	13±1	7±1	9±1
Peak Systolic Pressure (mmHg)	96±10	97±6	70±4	91±3*
Left Ventricular End Diastolic Pressure (mmHg)	4±1	7±2	59±4	54±4*
Left Ventricular Developed Pressure (mm Hg)	92±11	91±5	10±4	37±5*
LV $+dp/dt_{max}$ (mm Hg/s)	4035±753	3718±225	361±120	1261±210*

Preischemia indicates the value of various parameters at 50 min. Reperfusion indicates the value at 40 min.

*$P < 0.05$ compared to corresponding control value.

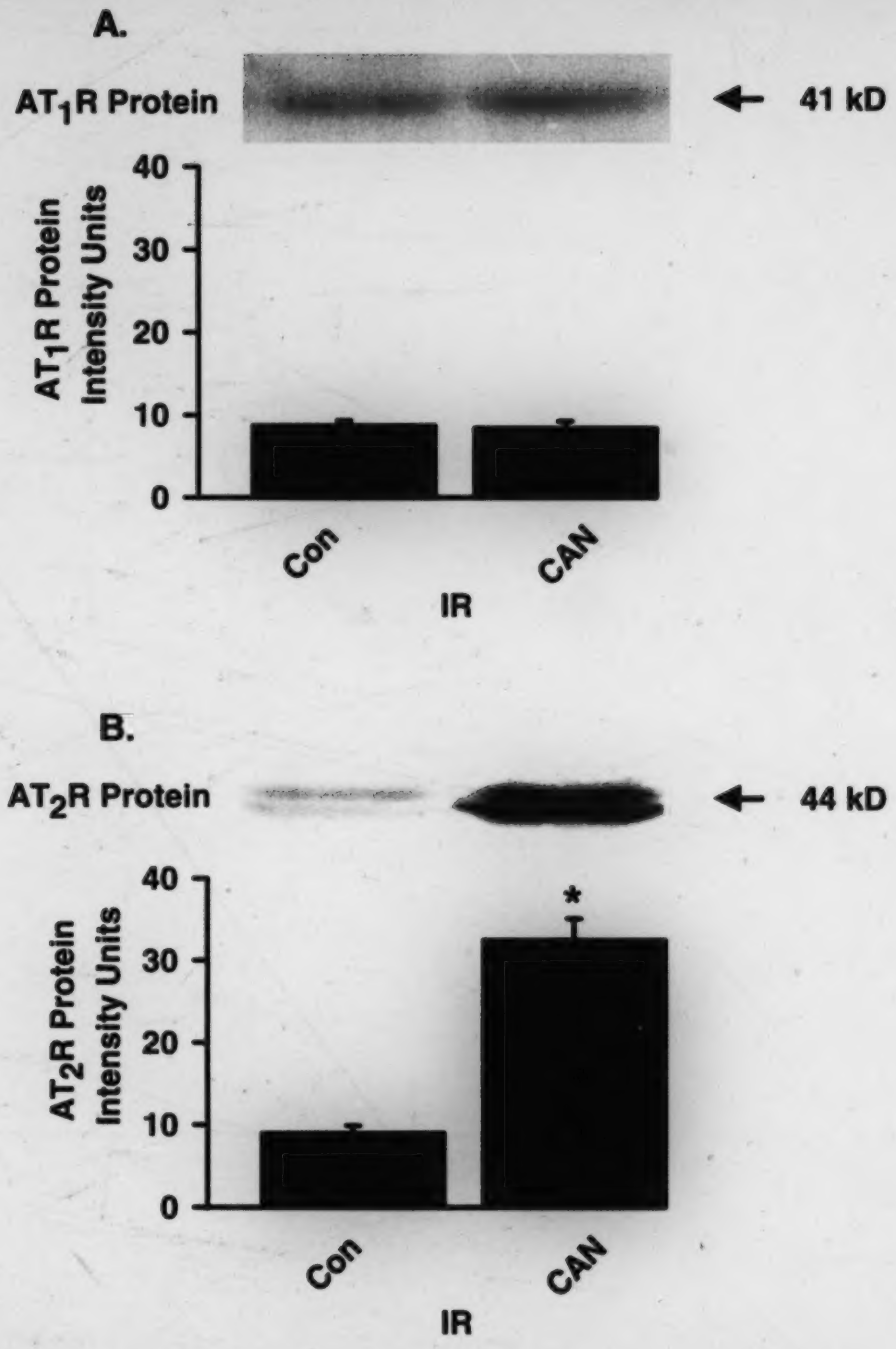

Figure 11 Effect of candesartan (CAN) on angiotensin II type 1 receptor (AT_1R) (panel A) and angiotensin II type 2 receptor (AT_2R) (panel B) protein content after ischemia-reperfusion (IR) in Langendorff preparation. *$p<0.01$ when compared to controls (Con)

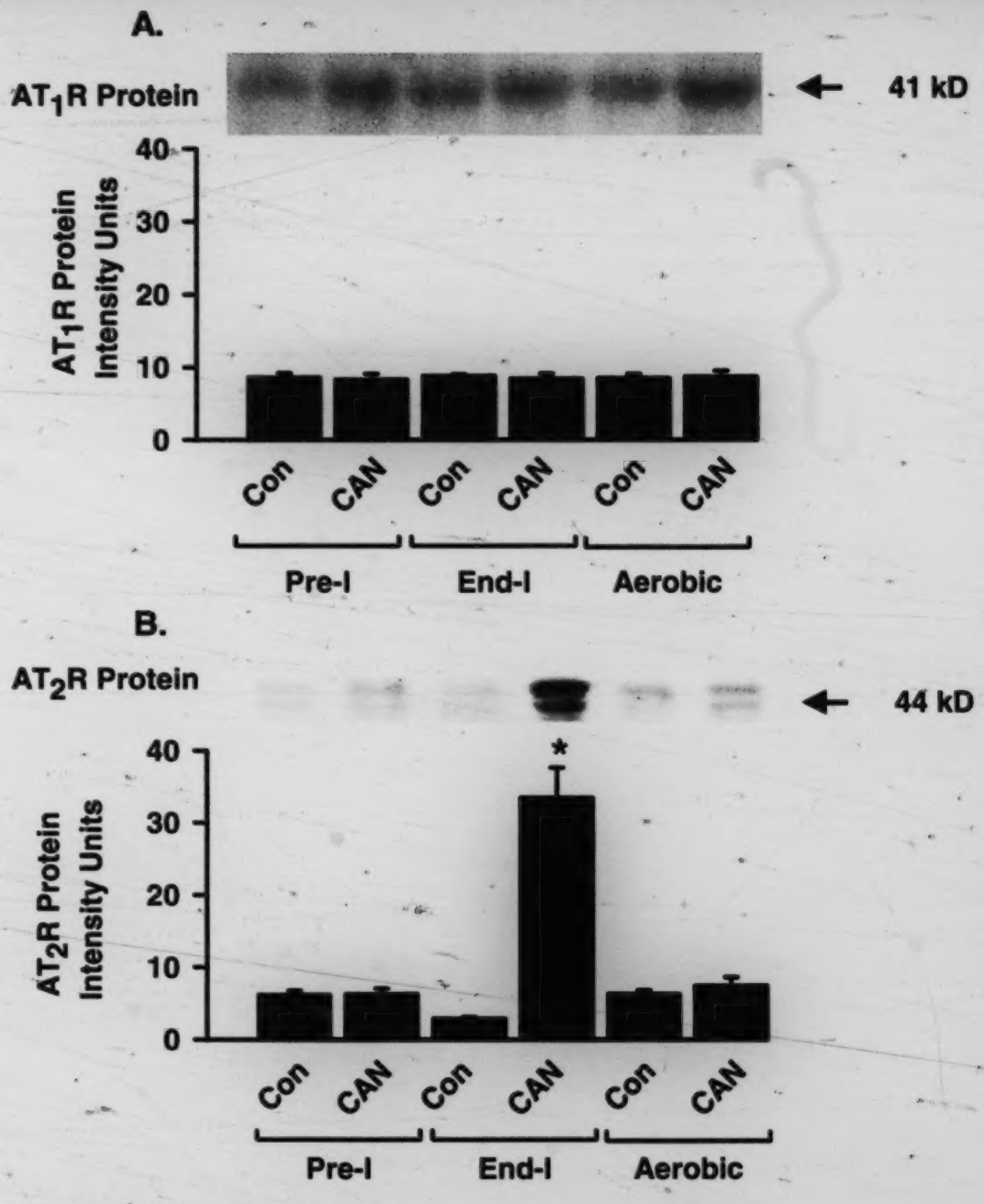

Figure 12 Effect of candesartan (CAN) on angiotensin II type 1 receptor (AT_1R) (panel A) and angiotensin II type 2 receptor (AT_2R) (panel B) protein content in Pre-I, End-I and Langendorff Aerobic hearts. *$p<0.01$ when compared to controls (Con).

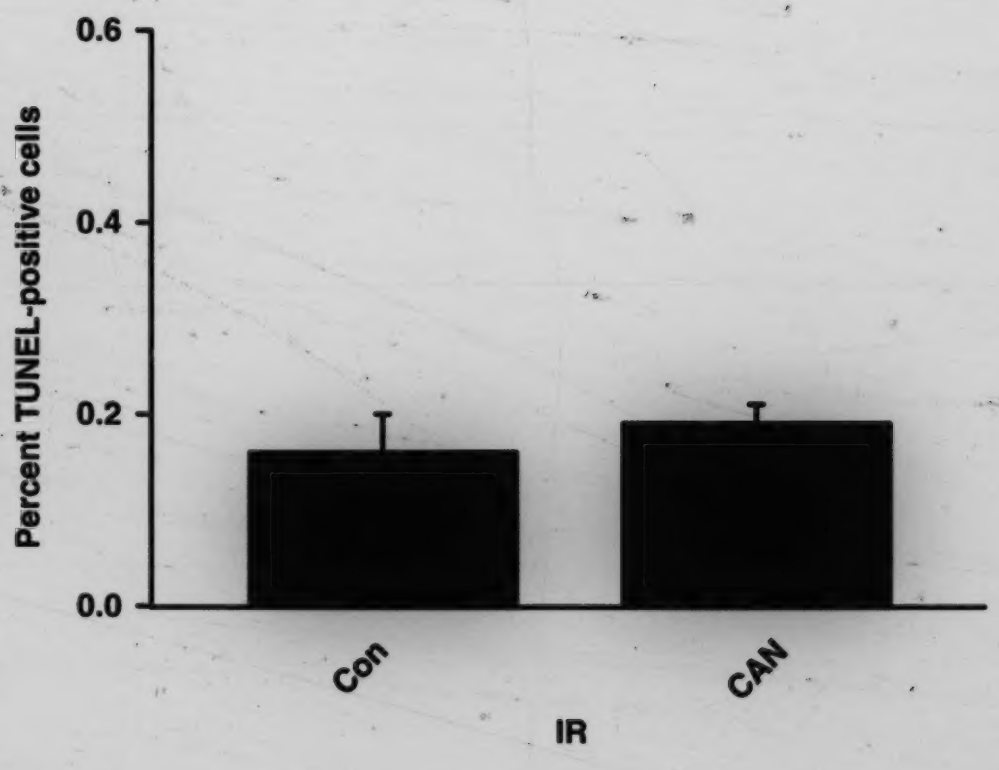

Figure 13 Effect of candesartan (CAN) on apoptosis in Landendorff-perfused hearts after ischemia-reperfusion (IR). Percent TUNEL positive cells= number of TUNEL positive cells among 1000 nuclei counted, and expressed as percent. Control (Con)

Table 4. Effects of Pretreatment With Candesartan on Apoptosis Under Different Protocols

	Control		Candesartan	
Protocols	TUNEL-assay	Early/Late	TUNEL-assay	Early/Late
Ischemia-Reperfusion	0.16±0.04	0.60±0.22	0.19±0.04	0.26±0.04
Pre-I	0.20±0.03	0.31±0.08	0.29±0.16	0.33±0.09
End-I	0.18±0.10	0.10±0.02	0.46±0.25	0.15±0.04
Aerobic	0.12±0.04	0.63±0.2	0.24±0.09	0.23±0.1

Values denote apoptotic index at the end of each protocol. Pre-I represents hearts at the end of 50 min baseline perfusion. End-I represents hearts, which underwent 50 min of baseline perfusion followed by 30 min of ischemia. Aerobic represents hearts subjected to 120 min of perfusion. Candesartan was administered for 40 min in baseline perfusion.

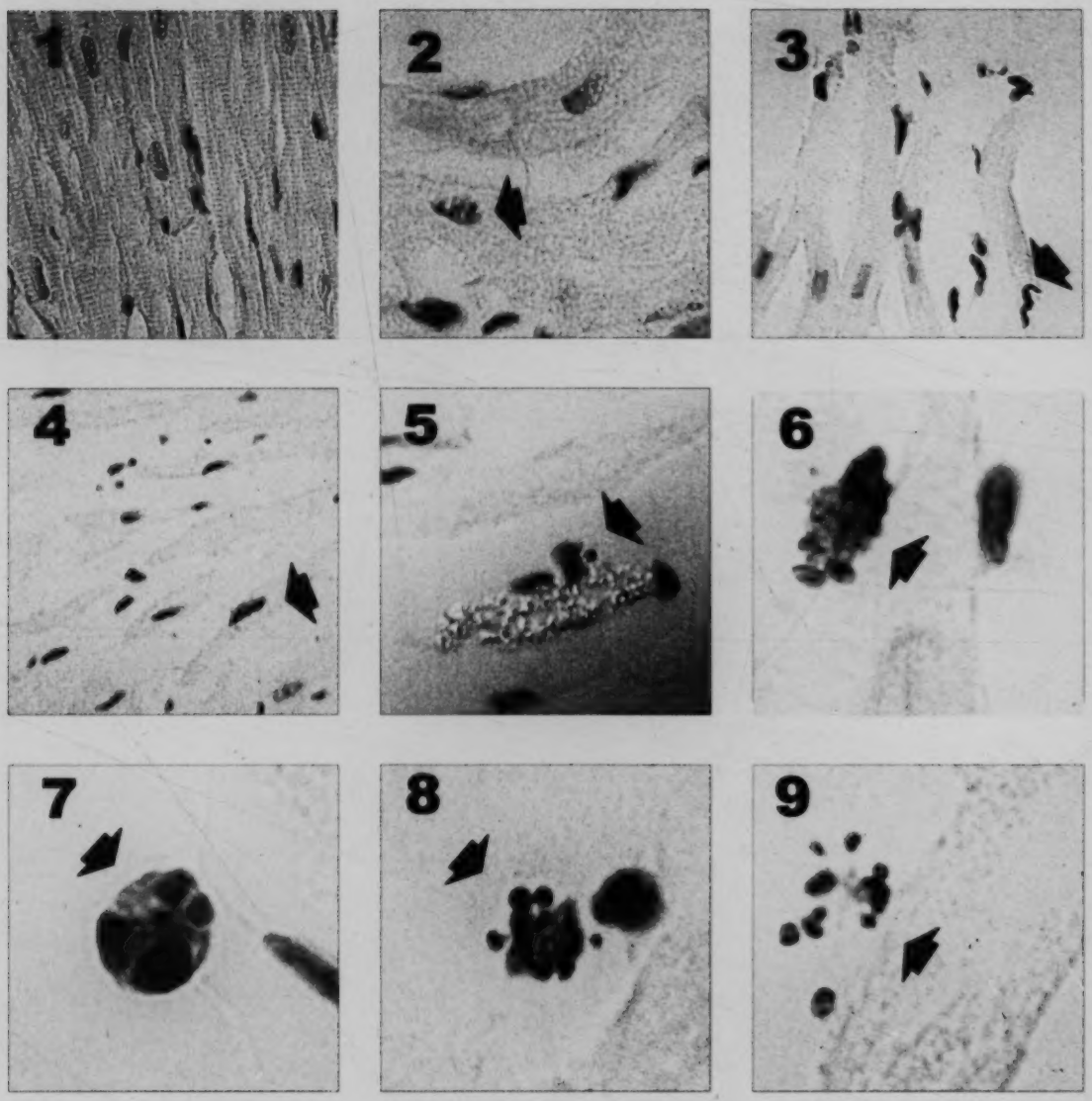

Figure 14 Nuclear morphology of apoptosis. 1. Normal Myocardium, 2. Partial Faint Labeling [category 1], 3-4. Pyknotic Strong Labeling [category 2], 5 Cytoplasmic Blebbing, 6-8. Progressive Stages of Nuclear Fragmentation [category 3], and 9 Apoptotic Body Formation [category 4].

B. EXPERIMENTS USING ISOLATED WORKING RAT HEART PREPARATION

Effects of AT_1R and AT_2R Antagonists on Mechanical Function

LV mechanical function, as judged by CO, LV work and CF, was stable in untreated hearts during the first 50 min period of aerobic perfusion. When hearts were subjected to global no-flow ischemia, mechanical function rapidly ceased (<1 min). Upon reperfusion, mechanical function resumed slowly and by the end of reperfusion CO and LV work had recovered to 68.0±4.2% and 62.9±4.6% (Figure 15 and 16; Table 5 and 6), respectively, of pre-ischemic values. However, during reperfusion CF recovered to pre-ischemic values (Table 5 and 6).

Pre-ischemic presence of CAN had no effect on mechanical function or CF during baseline perfusion. However, when these hearts were subjected to IR, CAN pretreatment caused a significant increase in post-ischemic recovery of CO (78.2±1.7%; $p<0.05$) and LV work (72.4±2.1%; Figure 15 and 16, Table 5 and 6; $p<0.05$). However, no effect on CF was observed.

Similarly, losartan had no effect on mechanical function or CF in baseline perfusion. However, losartan pretreatment showed no effect on post-ischemic recovery of CO (58.9±10.7%) and LV work (53.6±9.9%) when compared to untreated as well as CAN-pretreated rat hearts (Figure 15, Table 5). Moreover, no effect on CF was observed.

Meanwhile, PD, when present during baseline perfusion, had no effect on mechanical function or CF. When hearts were subjected to IR, PD pretreatment did not

alter the recovery of CO (65.9±9.7%) or LV work (61.7±9.8%) at the end of reperfusion (Figure 16, Table 6). Moreover, PD had no effect on recovery of post-ischemic CF.

However, when PD was co-administered with CAN, the improved recovery of CO (78.2±1.7%) and LV work (72.4±2.1%) observed with CAN was inhibited (PD+CAN; CO 63.4±3.8% and LV work 57.1±4.3%) at the end of the reperfusion, although there was no change in CF (Figure 16, Table 6; $p<0.05$).

In the groups of hearts subjected to prolonged Aerobic perfusion, untreated, CAN, losartan, PD, and PD+CAN showed stable CO and LV work for the first 75 min, thereafter a steady decline (~15%) in all the functional parameters were observed during the remaining 40 min in reperfusion that was similar in all 5 groups.

Effects of AT_1R and AT_2R Antagonists on AT_1R and AT_2R Protein Contents

The protein contents of AT_1R, that were determined in tissue frozen at the end of IR, were similar in untreated, CAN, losartan, PD and PD+CAN pretreated rat hearts (Figure 17).

However, protein levels of AT_2R were significantly increased with CAN pretreatment (Figure 18A and B; $p<0.05$). Similarly losartan pretreatment also increased the AT_2R protein contents in hearts subjected to IR (Figure 18A). Although, no change in AT_2R protein content was observed with PD pretreatment alone, PD+CAN pretreatment significantly attenuated the increased AT_2R protein content observed with CAN pretreatment (Figure 18B; $p<0.05$).

The protein contents of AT_1R and AT_2R that were determined in tissue frozen at the end of Aerobic perfusion were similar in untreated, CAN, losartan, PD and PD+CAN pretreated hearts (Figure 19 and 20).

Effects of AT_1R and AT_2R Antagonists on PKC_ε Translocation

PKC_ε protein contents were determined in both isolated membrane and cytosolic fractions. The ratio of membrane (particulate) to cytosolic fraction was used as an indicator of PKC_ε translocation.

Pretreatment with CAN significantly increased PKC_ε translocation in the hearts subjected to IR (Figure 21A and 21B). Similarly, losartan also significantly increased the PKC_ε translocation in the IR hearts. Pretreatment with PD per se did not affect the PKC_ε translocation fraction, however, PD was able to inhibit the CAN-mediated increase in PKC_ε content of the membrane fraction (Figure 21B). Interestingly, cytoplasmic PKC_ε content was also increased with CAN and losartan pretreatment, however, no such effect was observed in PD and PD+CAN pretreated groups.

Myocardial PKC_ε translocation was similar in all groups (untreated, CAN, losartan, PD, PD+CAN) at the end of prolonged Aerobic perfusion.

Effects of AT_1R and AT_2R Antagonists on cGMP Content

In hearts subjected to IR, cGMP content was significantly elevated in hearts pretreated with CAN or losartan (Figure 23A). Pretreatment with PD did not affect cGMP content by itself, but it inhibited the CAN-mediated increase in cGMP content (Figure 23B).

The myocardial content of cGMP was similar in all groups (untreated, CAN, losartan, PD, PD+CAN) at the end of prolonged aerobic perfusion (Figure 24A and 24B).

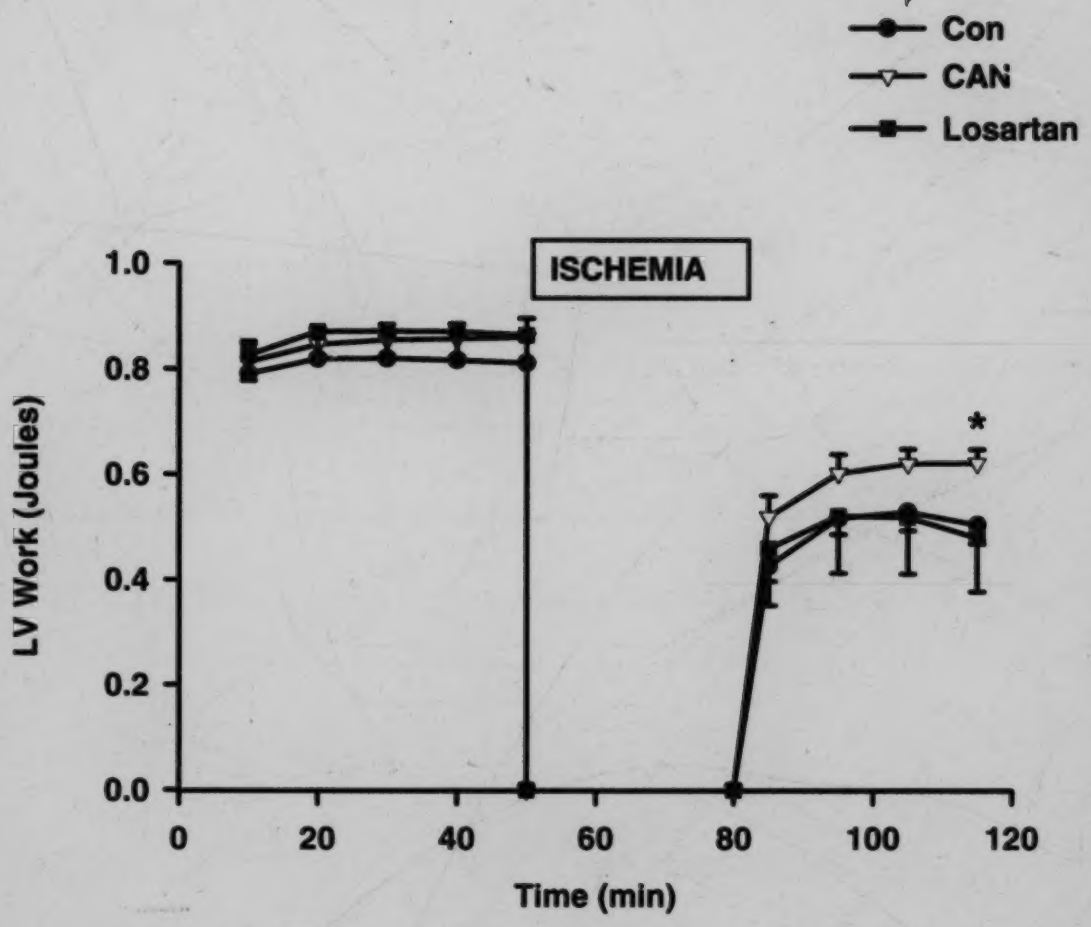

Figure 15 Effect of candesartan (CAN) and losartan on left ventricular (LV) work in isolated working rat hearts. *p<0.05 when compared to controls (Con).

Table 5. Effects of 40 min of Pretreatment With Candesartan (CAN) or Losartan on Recovery of Function After 25 min of Global Ischemia in Isolated Working Rat Heart Preparation

	Preischemia			Reperfusion		
Functional Parameters	Control (n=6)	CAN (n=7)	Losartan (n=7)	Control (n=6)	CAN (n=7)	Losartan (n=7)
Coronary Flow (ml/min)	19.6±2.2	18.9±2.1	21.6±2.3	19.7±0.62	21.0±1.4	19.9±3.6
Cardiac Output (ml/min)	61±3	63±2	65±3	41±3	49±2*	39±8
LV Work (Joules)	0.81±0.06	0.86±0.04	0.86±0.05	0.50±0.04	0.62±0.03*	0.48±0.10

Preischemia indicates the value of various parameters at 50 min. Reperfusion indicates the value at 40 min.

*P < 0.05 compared to corresponding control value.

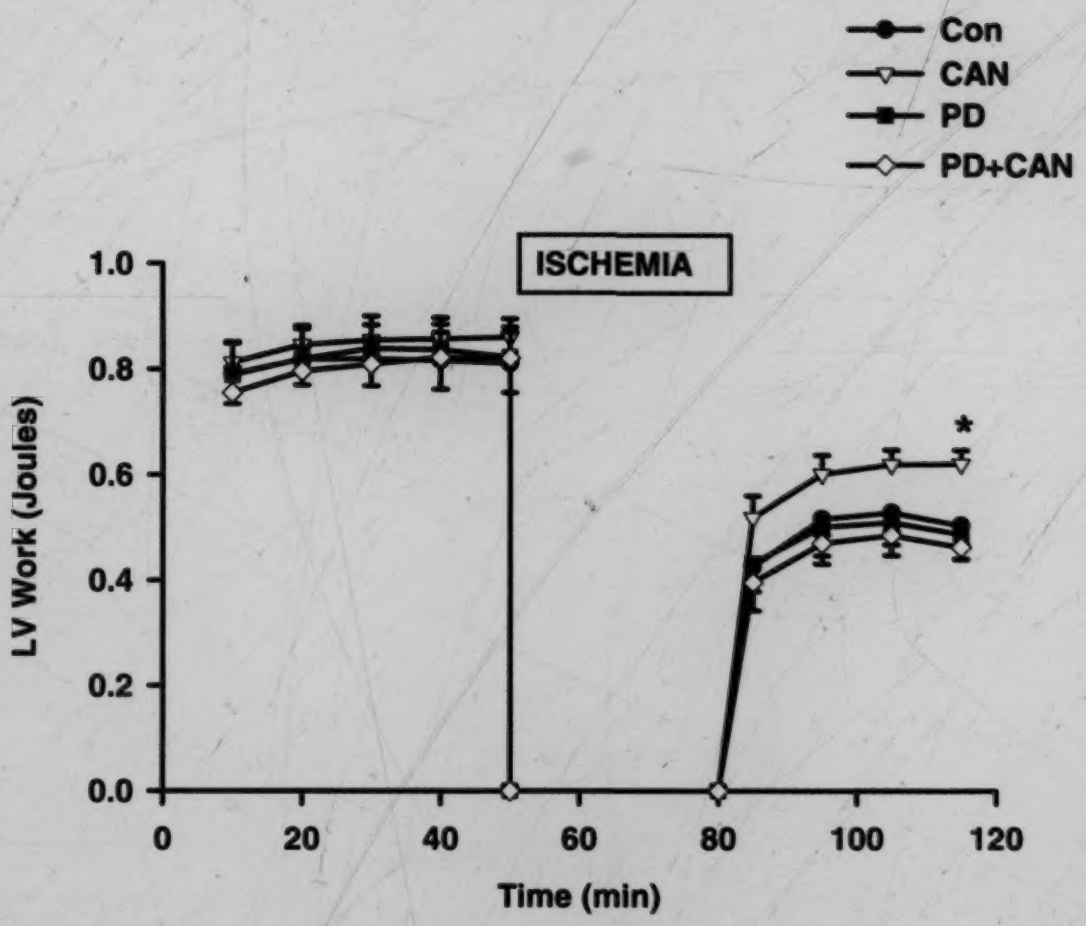

Figure 16 Effect of candesartan (CAN), PD 123,319 (PD) and their combination (PD+CAN) on left ventricular (LV) work in isolated working rat hearts. *$p<0.05$ when compared to controls (Con) and PD+CAN.

Table 6. Effects of 40 min of Pretreatment With Candesartan (CAN) or PD123,319 (PD) or the combination on Recovery of Function After 25 min of Global Ischemia in Isolated Working Rat Heart Preparation

	Preischemia				Reperfusion			
Functional Parameters	Control (n=6)	CAN (n=7)	PD (n=5)	PD+CAN (n=5)	Control (n=6)	CAN (n=7)	PD (n=5)	PD+CAN (n=5)
Coronary Flow (ml/min)	19.6±2.2	18.9±2.1	21.2±2.3	16.9±1.5	19.7±.0.62	21.0±1.4	21.0±0.9	19.5±1.7
Cardiac Output (ml/min)	61±3	63±2	64±3	63±3	41±3	49±2*	41±5	39±2
LV Work (Joules)	0.81±0.06	0.86±0.04	0.82±0.06	0.82±0.05	0.50±0.04	0.62±0.03*	0.49±0.05	0.46±0.02

Preischemia indicates the value of various parameters at 50 min. Reperfusion indicates the value at 40 min.

*P < 0.05 compared to corresponding control and PD+CAN.

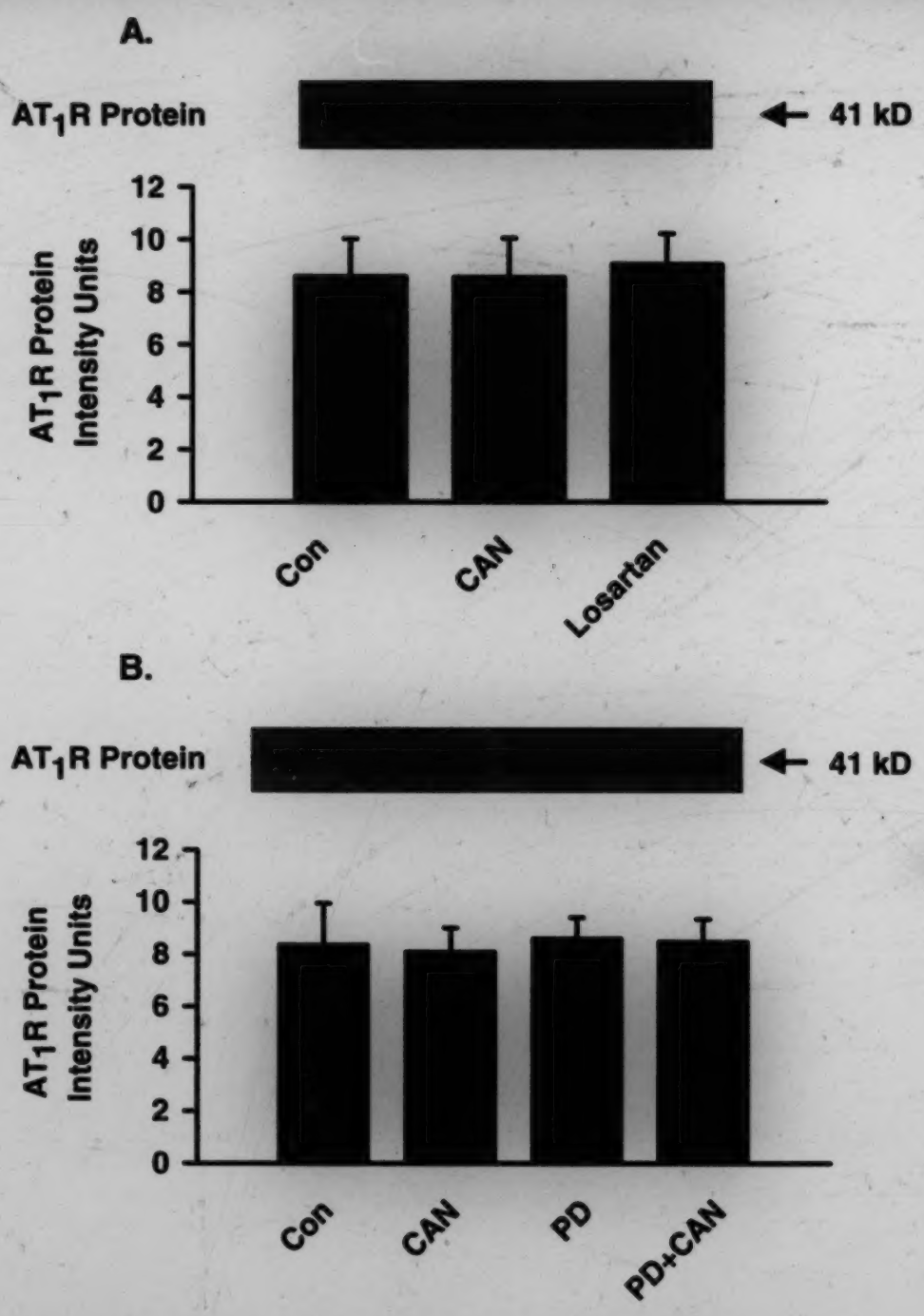

Figure 17 Effect of candesartan (CAN) and losartan on AT_1R protein (A) in hearts after ischemia-reperfusion. Effect of CAN, PD and their combination(PD+CAN) on AT_1R protein (B) in hearts after ischemia-reperfusion in isolated working rat heart.

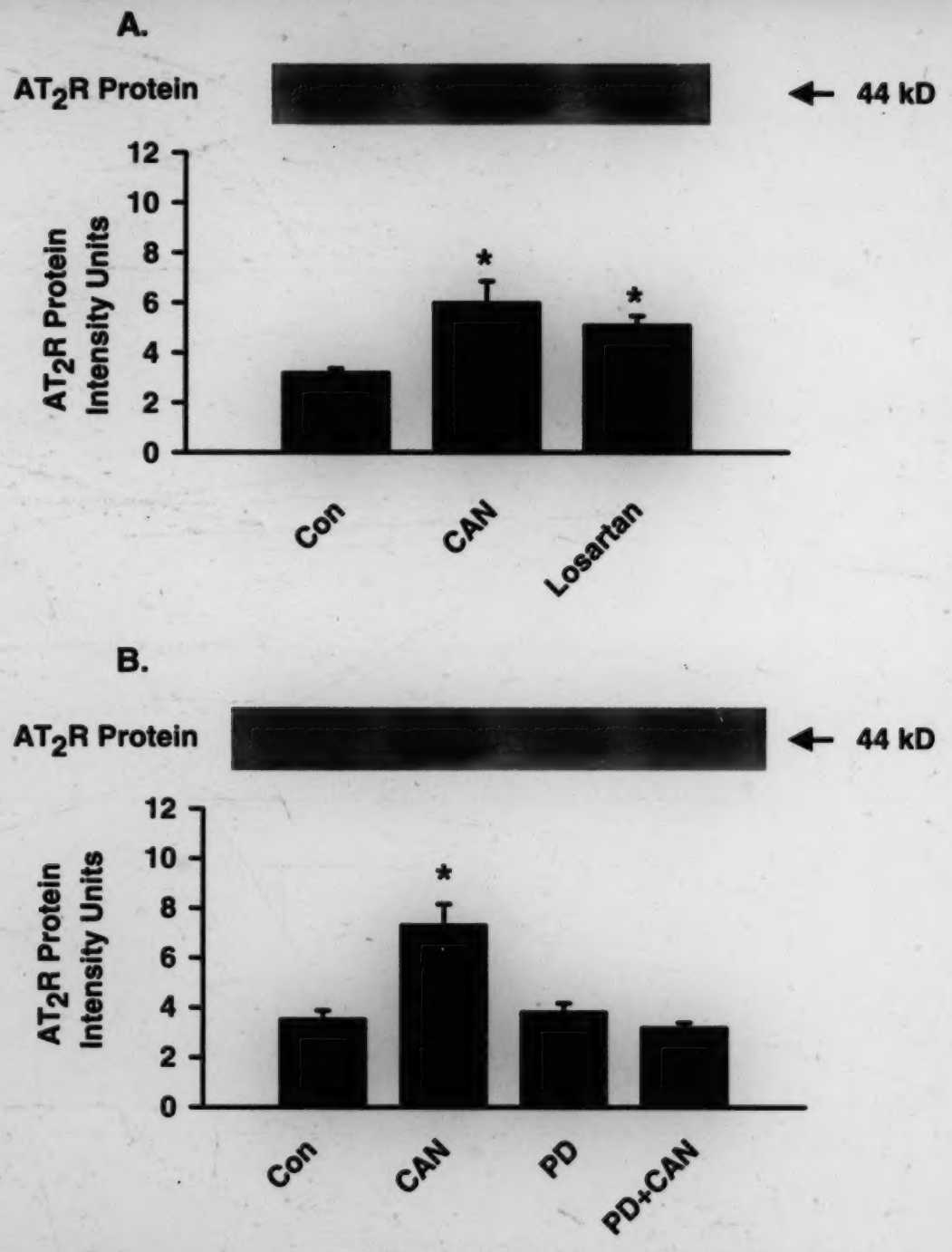

Figure 18 Effect of candesartan (CAN) and losartan on AT_2R protein (A) in hearts after ischemia-reperfusion. Effect of CAN, PD and their combination (PD+CAN) on AT_2R protein (B) in hearts after ischemia-reperfusion in isolated working rat heart.*$p<0.01$ when compared to controls (Con) and PD+CAN.

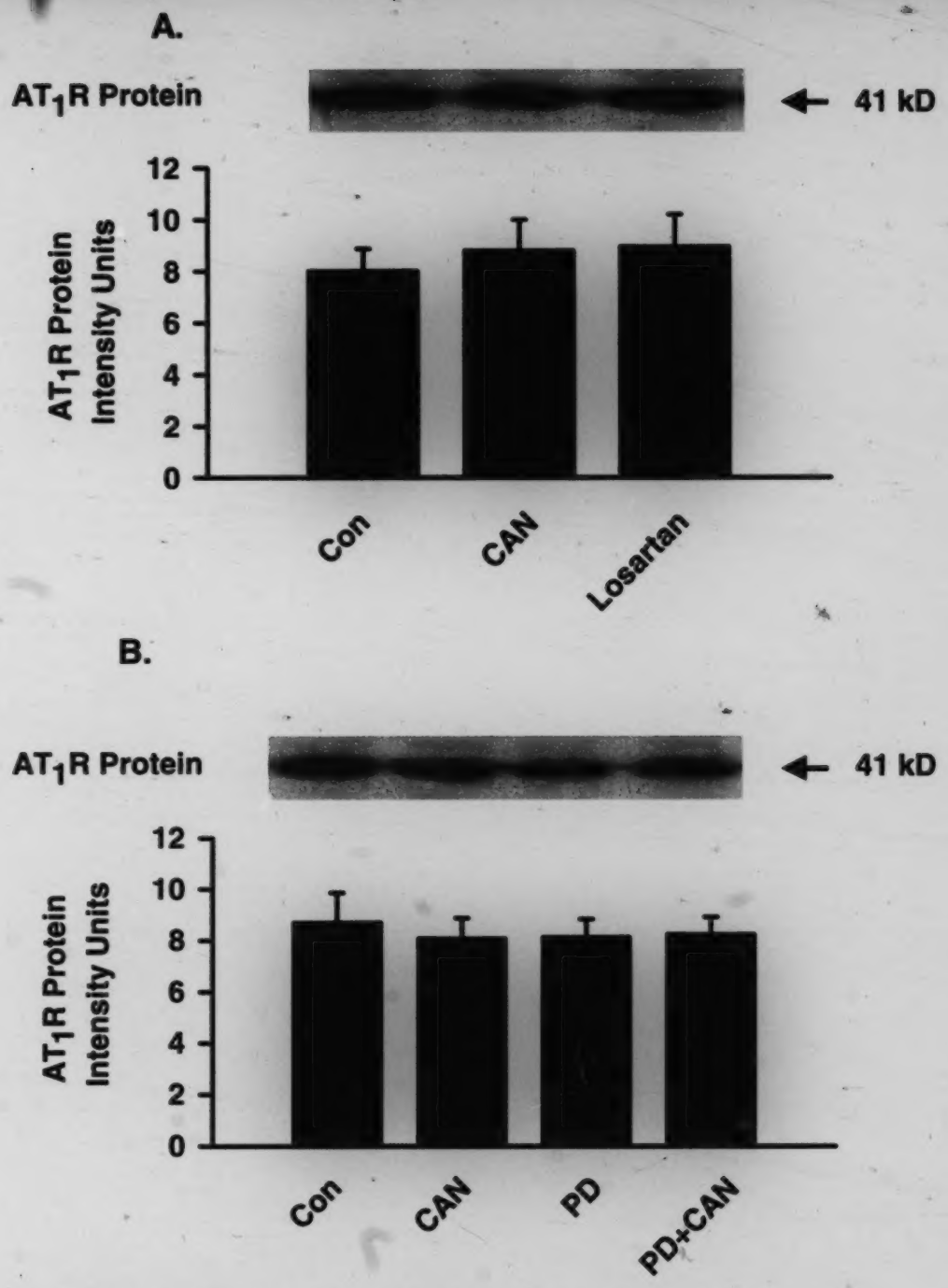

Figure 19 Effect of candesartan (CAN) and losartan on AT_1R protein (A) in hearts in aerobic protocol. Effect of CAN, PD and their combination (PD+CAN) on AT_1R protein (B) in hearts in aerobic protocol in isolated working rat heart. Controls (Con)

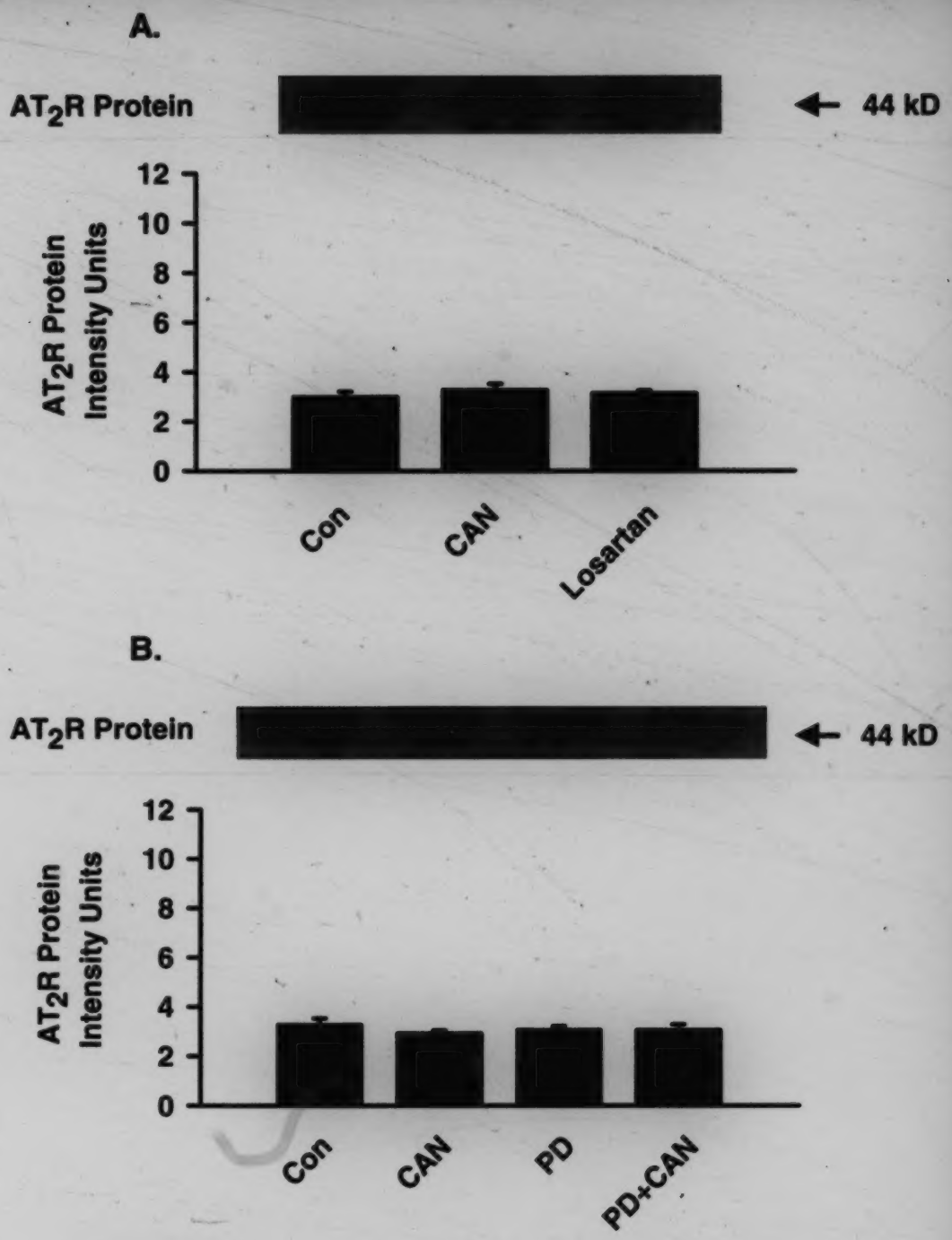

Figure 20 Effect of candesartan (CAN) and losartan on AT_2R protein (A) in hearts in aerobic protocol. Effect of PD and its combination (PD+CAN) on AT_2R protein (B) in hearts in aerobic protocol in isolated working rat heart. Controls (Con)

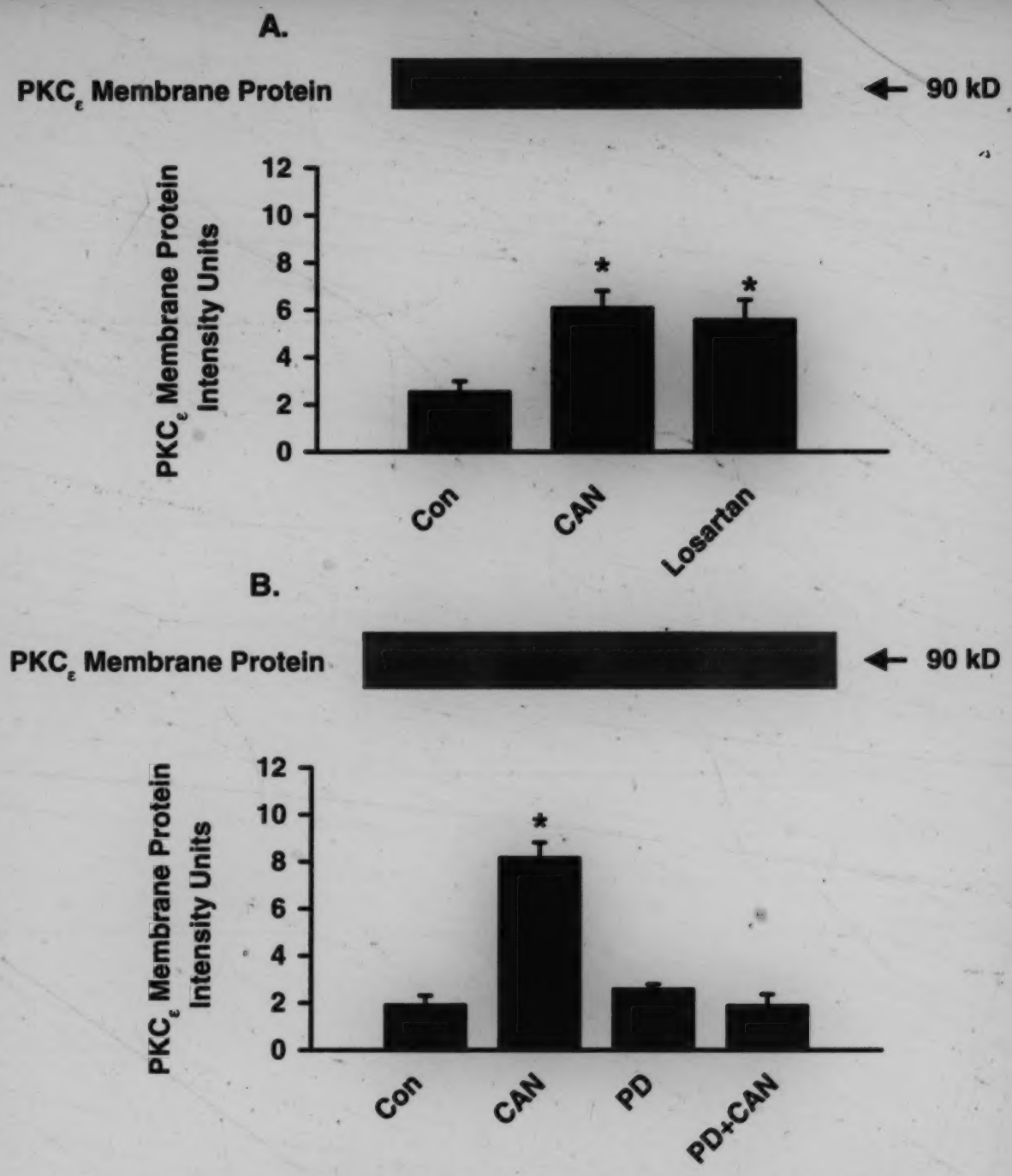

Figure 21 Effect of candesartan (CAN) and losartan on PKC_ε activation (A) in hearts after ischemia-reperfusion. Effect of CAN, PD and their combination (PD+CAN) on PKC_ε activation (B) in hearts after ischemia-reperfusion in isolated working rat heart. *$p<0.01$ when compared to controls (Con) and PD+CAN.

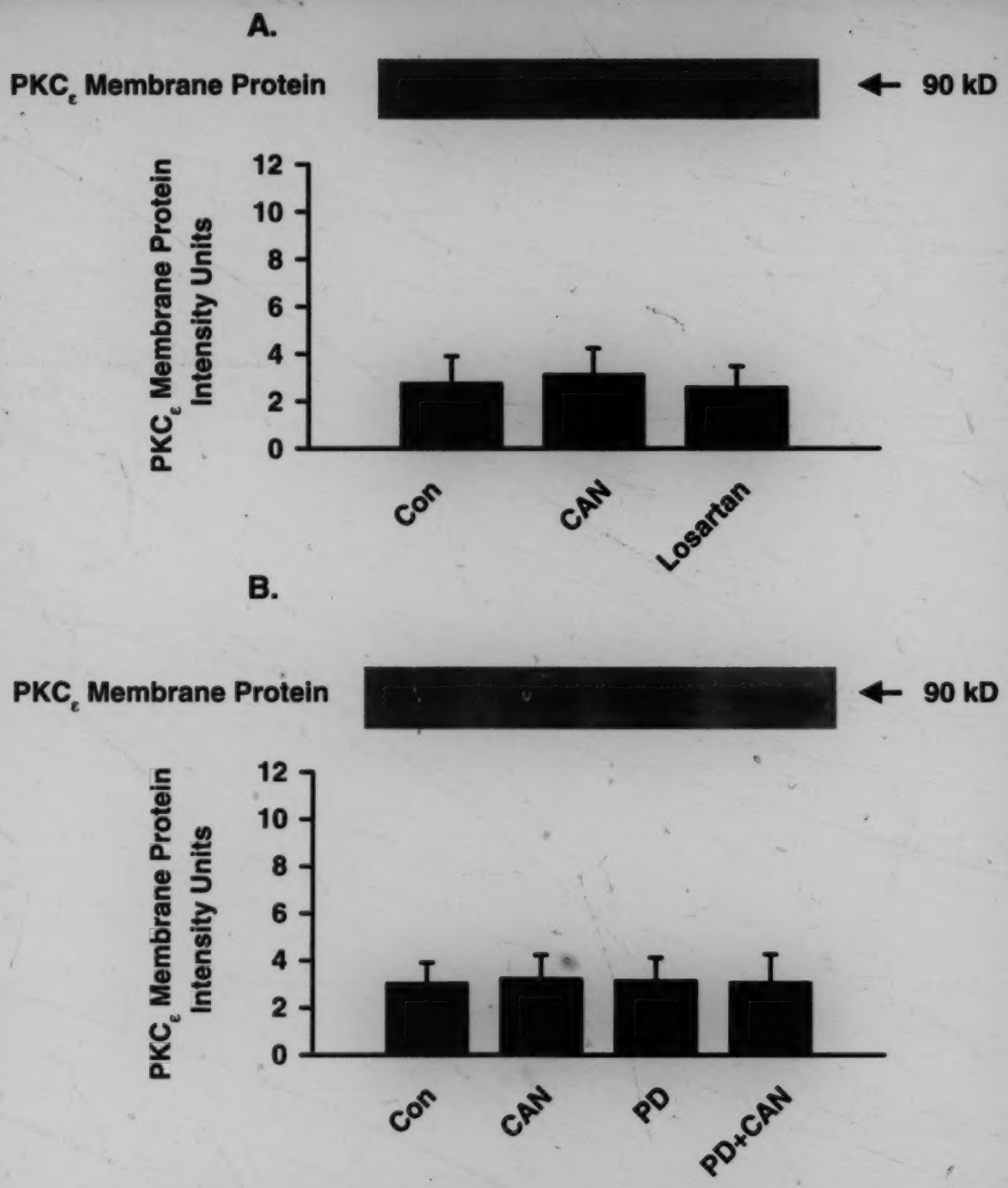

Figure 22 Effect of candesartan (CAN) and losartan on PKC_{ε} activation (A) in hearts after aerobic protocol. Effect of CAN, PD and their combination (PD+CAN) on PKC_{ε} activation (B) in hearts after aerobic protocol in isolated working rat heart. Controls (Con)

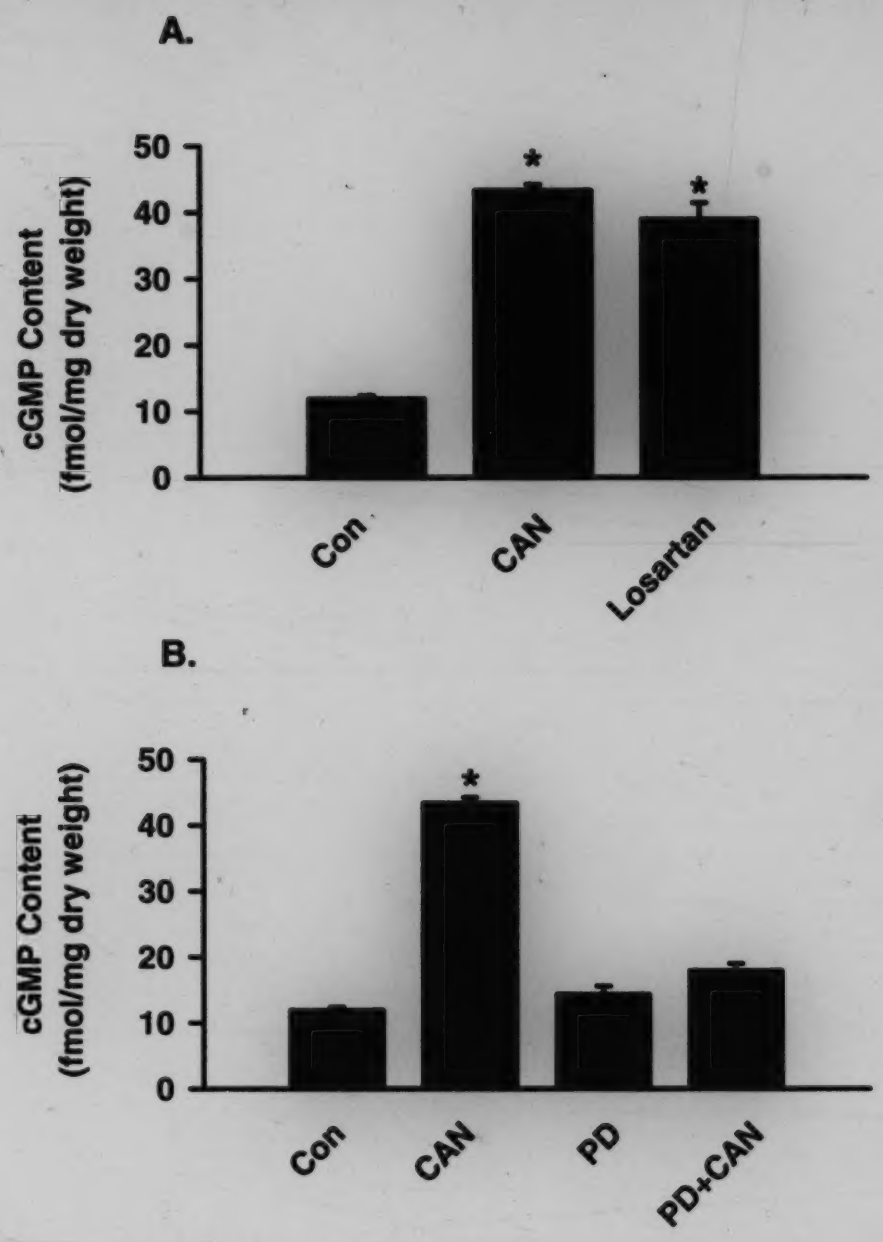

Figure 23 Effect of candesartan (CAN) and losartan on myocardial cGMP content (A) in hearts after ischemia-reperfusion. Effect of CAN, PD and its combination (PD+CAN) on myocardial cGMP content (B) in hearts after ischemia-reperfusion in isolated working rat hearts.*$p<0.01$ when compared to controls (Con) and PD+CAN.

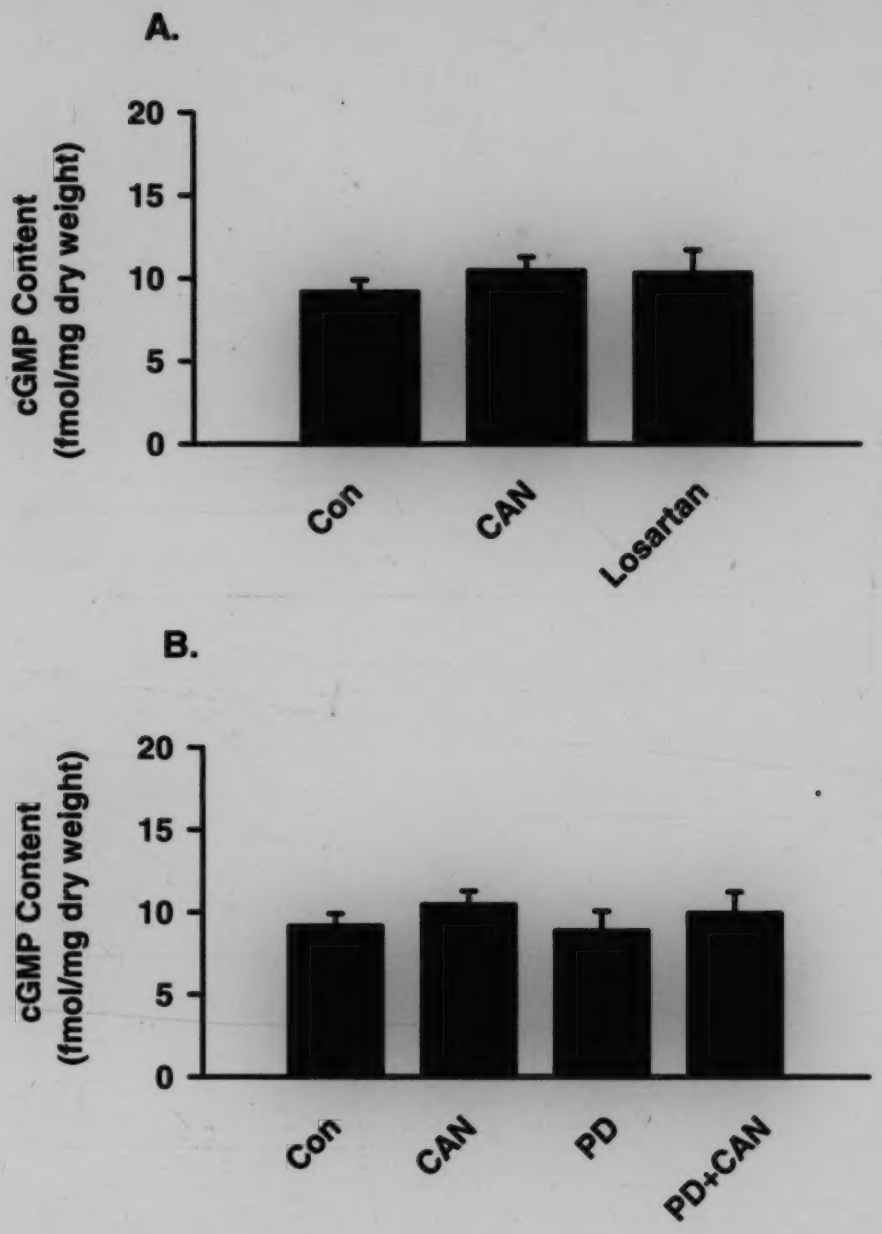

Figure 24 Effect of candesartan (CAN) and losartan on myocardial cGMP content (A) in hearts after prolonged aerobic protocols. Effect of CAN, PD and their combination (PD+CAN) on myocardial cGMP content (B) in hearts after control protocol in isolated working rat hearts. Controls (Con)

DISCUSSION

The present study shows that AT_1R blockade with CAN induces cardioprotection in acute IR. This cardioprotection is partially due to AT_2R activation as inhibition of AT_2R with PD attenuated the cardioprotective effect. Furthermore, AT_2R activation was also associated with increased cGMP production and PKC_ε activation implicating these factors in cardioprotection. Pretreatment with CAN also increased AT_2R protein content which might be due to Ang II binding to this receptor (see below). The inability of CAN to affect apoptosis suggests that AT_2R activation has no effect on this pathway. Similarly, losartan also increased AT_2R protein content and increased cGMP production and PKC_ε activation suggesting a class effect of AT_1R blockade rather than drug-specific (CAN) effect. However, unlike CAN, losartan did not improve post-ischemic functional recovery, suggesting that the discordant results may be related to the difference in their pharmacodynamic properties.

Effects of AT_1R Blockade on Functional Recovery after IR

Candesartan and Losartan

The present study showed that CAN mediates a cardioprotective effect as measured by improved recoveries of LVDP and LV +dp/dt. However, no effect on coronary flow was observed signifying that CAN-mediated cardioprotection was mediated directly on cardiac muscle. Moreover, the observed effect with CAN required greater than 5 min pretreatment, as CAN, when present for only 5 min proior to ischemia had no effect on

post-ischemic recovery. This is in concordance with previous studies that have shown that 30 min of pretreatment with CAN is required in order to achieve a cardioprotective effect. Similarly, CAN also elecited a cardioprotective effect in hearts perfused in working mode. However, losartan, another AT_1R antagonist, did not possess any effect on post-ischemic functional recovery. Although, CAN results are in concordance with the previously published studies, the data on losartan further adds to the complex profile of activity in affecting functional recovery after IR.

Losartan was the first orally active AT_1R antagonist that was developed for clinical use and is therefore the most studied compound of its class. Over the years, however, many reports have been published which cumulatively have painted an inconclusive picture of effects of losartan in acute IR. In this respect, most of the studies with losartan have showed cardioprotection, however, others have presented results to the contrary. Specifically some studies have shown that losartan decreases ischemia-induced arrhythmia [116], increases coronary flow [117], increases cardiac output [117] and increases contractile function as measured by LV +dp/dt in post-ischemic hearts [118]. Moreover, losartan-induced reductions in infarct size have been reported [150]. In contrast, studies done in our laboratory have shown that losartan has deleterious effects on post-ischemic functional recovery [132, 133]. Also, numerous studies has shown that losartan have no effect on infarct size [135-137]. On the other hand, CAN has shown a consistent profile characterised by increased post-ischemic recovery [121, 138] and decreased infarct size [100, 151] in hearts subjected to IR. CAN has been shown to reduce IR-mediated cardiac dysfunction in rats [121] and pigs [100, 151] and is frequently associated with limiting infarct size [50, 100]. Difference between the cardioprotective efficacy of CAN and losartan may be

due to the marked difference in potency (daily dose recommended for CAN is 8-16 mg versus losartan 50-100 mg), affinity (CAN binds 80 times more readily to AT_1R than losartan) and dissociation rate (CAN $t_{1/2}$= 66 min versus losartan $t_{1/2}$= 2.5 min). Hence, it might be possible that these differences explain the different recoveries of post-ischemic function observed with the two antagonists [152].

Careful analysis of our results suggests that CAN is better than losartan in its protective effect on post-ischemic functional recovery when compared to matched controls, although both of the drugs belong to the same class of antagonists. This implies that perhaps CAN is able to achieve a more complete blockade of AT_1R and that this effect might have affected the outcome on post-ischemic contractile recovery. Since losartan can be easily displaced from its receptor site and it was not present during reperfusion, local Ang II production during IR might still have potentially stimulated AT_1R. These findings are substantiated by a study done by Wang *et al.* [138] who showed that a 10 min washout period was enough for displacing losartan from its receptor site, while such an effect was not observed with CAN even after 90 min. This suggests that CAN exerts a more prolonged blockade of AT_1R than losartan, which might have resulted in the different outcomes. In this context, it is possible that during IR, Ang II levels are increased in the intersitial space to an extent such that Ang II can competitively remove losartan from its binding site. Under these circumstances, acute activation of AT_1R would precipitate deleterious effects (perhaps via PKC_α mediated Ca^{2+} intracellular increase [33-35] and resulting Ca^{2+} overload) to a point that the cardioprotective effect achieved by AT_2R activation (as discussed below) via AT_1R blockade will be negated. However, this is not possible with CAN as it is an insurmountable AT_1R antagonist and

hence in IR, Ang II increase in intersitial space would have no effect on the antagonistic properties of CAN. Therefore, CAN mediates its cardioprotective effect by virtue of its insurmountable nature of AT_1R.

A previous study done in a Langendorff perfused hearts also examined the effects of both of these AT_1R antagonists [138]. That study showed a significant increase in post-ischemic functional recovery with both CAN and losartan when compared to matched controls. The difference in effect between the present and the previous study might be due to the concentration used or in the treatment protocol. However, the improvement in post-ischemic contractile recovery in the presence of CAN when compared to matched controls, and the non-significant differences between CAN and losartan in terms of recovery after IR are still in agreement. Nevertheless, CAN showed a beneficial effect on post-ischemic functional recovery, while losartan did not show a similar activity profile in our experiments.

In addition, the recovery profile observed with losartan in the experiments was contrary to the findings previously published by Ford *et al* [132, 133] from the laboratory. The studies done by Ford *et al* showed that administration of losartan for 5 min before ischemia, during 30 min of ischemia and for 40 min of post-ischemic reperfusion decreases LV work and myocardial efficiency. This is in contrast to the present study that found that losartan has no effect on post-ischemic functional recovery. The length of losartan administration (40 min pretreatment in present study versus 5 min in Ford *et al*) and the time of ischemia (25 min in the present study versus 30 min in Ford *et al*), might have caused discrepancies between our results and previous findings from our laboratory.

Candesartan, PD and their Combination

The present study demonstrated that the CAN-mediated improvement in post-ischemic function can be partially attributed to AT_2R activation. When PD was co-administered with CAN, the CAN-induced cardioprotective effect was attenuated. However, PD by itself, had no effect on post-ischemic functional recovery.

Most of the studies on AT_1R antagonists attributed their therapeutic benefits to their antagonistic properties on AT_1R. However, the idea of AT_1R blockade resulting in Ang II binding to AT_2R, and thereby enhancing synthesis and release of bradykinin was first proposed by Wiemer *et al* [153]. Since then, the findings of numerous studies have supported this hypothesis and it is now becoming increasingly evident that the beneficial effect of AT_1R blockade can be partially attributed to unopposed activation of AT_2R, thereby increasing bradykinin, NO and cGMP [79-82, 100, 101, 154, 155].

The involvement of non-AT_1R mediated cGMP production was known previously. It was shown that infusion of Ang II stimulated guanylate cyclase and thereby produced cGMP in aortic segments [154]. Further analysis demonstrated that Ang II concentration-dependently increased cGMP production [153, 155] and this effect of Ang II mediated on cGMP production was blocked by a bradykinin B_2 antagonist and a NO synthase inhibitor [154]. This role of NO, although preliminary, was later confirmed by Seyedi *et al.* [79] who established that in fact AT_2R activation produces NO as measured by nitrate and nitrite levels in isolated coronary vessels. The effect of Ang II-induced NO release was mediated by local kinin production, as it was shown that a bradykinin B_2 receptor antagonist inhibited this event, establishing B_2 receptors as potential trigger for NO production [101].

However, it was Liu *et al.*, who extended this pathway from vasculature to the heart. Using 2 month old MI as a heart failure model, AT_1R antagonist treatment for the following two months after MI showed beneficial effects, functionally, as well as morphologically [82]. These events were subsequently inhibited by AT_2R blockade and B_2 receptor antagonists, establishing the key role they play in cardioprotection [82]. Recent observations further extended this idea in acute infarction studies where beneficial effects of CAN were abrogated by AT_2R and B_2 receptor antagonists suggesting that the cardioprotective effect of CAN involves activation of AT_2R and bradykinin release [100]. However, whether these protective mechanisms of CAN are important for decreasing reperfusion-induced functional damage in the isolated hearts was not determined.

The present study is the first to establish that during acute IR, the CAN-mediated cardioprotective effect is partially due to AT_2R activation. In support, when PD was infused in combination with CAN, the protective effect mediated by CAN was attenuated, implicating AT_2R activation as the cardioprotective mechanism. Furthermore, this study is also the first to show that unopposed activation of AT_2R can take place by Ang II produced locally. Since most of the vascular studies involves Ang II infusion [79, 80, 101] and subsequent myocardial studies were done *in vivo* [82, 100] (where the heart is exposed to both plasma and cardiac Ang II), involvement of intracardiac Ang II was not clear. As this study was performed in isolated working rat heart, any Ang II involved in this process must be due to local production of Ang II, suggesting the role of intracardiac Ang II in AT_2R activation [15, 126].

Effects of AT_1R blockade on AT_2R Protein Content after IR

Besides AT_2R activation, AT_2R protein levels were increased in CAN pretreated hearts. The present study showed that when hearts are pretreated with CAN in both Langendorff and working mode, AT_2R protein content increased. This increase was also evident in losartan-treated hearts suggesting a class effect rather than drug (CAN) specific effect. Moreover, this increase was inhibited by co-administration of PD suggesting that locally produced Ang II might be involved in the increase in AT_2R protein content. This has also been confirmed in our laboratory in experiments involving canines subjected to IR. The AT_1R and AT_2R findings in this study differ from those of Yang *et al.*, who did not detect an increase in AT_2R protein content with losartan [122]. This discrepancy might be due to differences in methodology, as the present study used immunoblots for the proteins (instead of autoradiography), timing of IR, as well as the different pharmacodynamic properties of these two drugs.

Regulation of AT_2R expression has been a subject of interest in the past few years and some of the earliest observations were made in cell culture studies. Preliminary attempts to detect AT_2R in adult rat cell culture proved to be inconclusive and researchers were divided on whether AT_2R were present [156-158] or not [159, 160]. In this respect, autoradiographic studies (studies looking at the receptor distribution by radioligand binding) approaches showed that AT_2R were present in adult rat myocardium, while *in situ* hybridization showed the opposite [161]. It was subsequently demonstrated that the amount of AT_2R expressed in *in vivo* rat hearts varied from the amount of AT_2R detected in cell cultures [162], suggesting that AT_2R is downregulated *in vitro* [163]. In support of this theory, it was later found that growth factors (such as platelet derived growth factor and

basic fibroblast factor) present in cell culture medium were potent in their ability to downregulate AT_2R [73]. This suggests that under acute experimental conditions, AT_2R can undergo rapid changes in its protein content in response to external stimuli.

In the same context, a study by Dudley and Summerfelt [164] showed that ligand occupation of AT_2R by Ang II effectively enhanced the cell surface AT_2R content. These observations were further extended to other AT_2R ligands, as Ang I, III and CGP 42112 also increased membrane AT_2R content [165]. Furthermore, when fetal human kidney cells (HEK293 cells) were examined for this phenomenon, similar findings were made [165]. Time course analysis showed that following addition of [^{125}I]-Ang II to R3T3 cells, a steady increase in specific AT_2R binding was observed and achieved maximum around 90 min, when the increase in AT_2R was approximately 8-fold [165]. These findings have led to the proposal that perhaps in situations of continuous AT_2R turnover, ligand occupancy of the receptor results in formation of a stable complex of receptor and ligand such that degradation is inhibited. This would result in the accumulation of AT_2R, and thus increase the total number of AT_2R in response to ligands.

Inhibition of receptor degradation might be the reason of increased AT_2R in this study. When the hearts are pretreated with CAN, it is possible that the increase in available Ang II (due to ischemia) in the intersitial space would result in Ang II binding to AT_2R (as AT_1R are not available). This binding of Ang II to AT_2R would make a stable complex of receptor and ligand, thereby inhibiting the degradation process of AT_2R and increasing AT_2R protein content. This is further supported by the fact that when PD was co-administered with CAN, the increase in AT_2R protein content was attenuated implying that Ang II binding to AT_2R might be involved in increasing AT_2R

protein content. Hence, Ang II might be the cause of the increase AT_2R protein content during CAN pretreatment in IR setting.

Despite the proposed mechanisms, studies looking at myocardial Ang II levels in IR are lacking. Studies done in hypertrophied rat hearts have shown that indeed Ang II is elevated during IR. Comparison of Ang II levels before ischemia and after IR indicated that IR increased Ang II content [126]. Since this study employed an isolated working rat heart preparation, increases in AT_2R could be due to local Ang II production. Previously Lindpaintner *et al.* showed that in isolated perfused rat hearts, the heart is capable of producing its own Ang II [15]. It was found that during aerobic perfusion, Ang II is released. Although, release during the first 50 min of perfusion is primarily due to sequestered precursors, release of Ang II following 50 min of perfusion in isolated perfused hearts is due to the local synthesis of Ang II. Taken together, this suggests that under pathological conditions where Ang II is increased (probably by local production), Ang II can act as a potential endogenous ligand for AT_2R, which then inhibits AT_2R degradation. This is further supported by the findings in the current study, which showed that the increase in AT_2R protein occurs only under conditions of pretreatment of CAN in combination with ischemia. Since under aerobic conditions, Ang II might be generated only minimally, a low amount of Ang II in the myocardium will not be expected to have a significant effect so that no increase in AT_2R is seen. However, during ischemia, Ang II might increase. When AT_1R is available, the total Ang II pool is distributed between these two receptor subtypes (AT_1R and AT_2R). Since the population ratio of AT_1R and AT_2R in *in vitro* conditions is 4:1, only a small amount of Ang II will be available for AT_2R [123]. However, when ischemia is coupled with CAN pretreatment, the available Ang

II will be expected to shift and bind to AT_2R, thereby inhibiting its degradation. The 4-fold increase in AT_2R found in this study occurred within a reasonable period, since the authors of a previous study showed an approximate 8-fold increase in receptor population in 90 min [165]. Thus, the increase in AT_2R observed in this study might be due to a CAN-mediated Ang II shift to AT_2Rs. However, further studies addressing the potential role of cardiac Ang II in increasing AT_2R levels are clearly warranted.

One other possible explanation of AT_2R upregulation is intracellular cross-talk. Studies have shown that AT_1R and AT_2R engage in cross-talk. Specifically, it has been reported that AT_2R stimulation in cultured rat neonatal cardiomyocytes and fibroblasts inhibited AT_1R dependent growth. Also, Ohkubo *et al.* [64] demonstrated that AT_2R is re-expressed in cardiac fibroblasts in fibrous regions in failing cardiomyopathic hamster hearts. This increased AT_2R activation, in turn, exerted an anti- AT_1R action on the progression of intersitial fibrosis during cardiac remodeling by inhibiting both fibrillar collagen metabolism and growth of cardiac fibroblasts. Furthermore, Horiuchi *et al* [53] examined the effect of Ang II on apoptosis in VSMC and reported that AT_1R stimulation inhibited the onset of apoptosis-induced by serum depletion. Conversely, under similar conditions the same authors found an enhanced apoptosis in AT_2R-transfected VSMC, suggesting a negative cross-talk occurring in apoptosis by two different receptors by the actions of the same ligand [53].

It was also shown that the stimulation of AT_2R in AT_2R cDNA transfected rat adult VSMC inhibited AT_1R-mediated tyrosine phosphorylation of STAT-1, 2 and 3 without any influence on Janus Kinase (an upstream activator of STAT) [166]. Later studies done by Nakajima *et al* [99] directly correlated decreased MAPK activity and subsequent

reduced DNA synthesis by AT_2R transfection. This negative cross-talk of mitogenesis between AT_1R and AT_2R is now confirmed in proliferating endothelial cells and microvascular angiogenesis [167]. At the functional level also, such as in blood pressure regulation, AT_2R has been shown to counteract the vasoconstrictive response of AT_1R. Hence, numerous lines of evidence exist to indicate that AT_1R and AT_2R engage in negative cross-talk [167].

Recent studies have shown that activation of PKC and mobilization of intracellular Ca^{2+} downregulate AT_2R expression [168]. It was found that the PKC-Ca^{2+} pathway affects the stability of AT_2R mRNA as well as the transcription rate of the AT_2R gene [168]. Since one of the primary downstream molecules of AT_1R is PKC [33-35], it is possible that during IR, an increase in Ang II in the intersitial space might activate AT_1R and thus PKC-Ca^{2+} mobilization, resulting in AT_2R mRNA downregulation. Thus, downregulation in mRNA might translate into lower AT_2R protein expression. However, in the presence of CAN, this activation of PKC is inhibited. This would, in turn, result in increased stability of mRNA as well as an increase in AT_2R transcription rate such that an increase in AT_2R protein is seen with CAN administration. Thus, the possibility exists that AT_1R and AT_2R engage in cross-talk with the intracellular second messengers that regulate the levels of receptor protein. However, given the fact that increases in AT_2R mRNA were first seen after 6 hours, the possibility of these mechanisms being involved in regulation of protein expression acutely is highly unlikely. Furthermore, aerobic hearts, which were exposed to CAN for 40 min, showed no effect on AT_2R protein content, questioning the validity of this hypothesis in this IR setting. The involvement of the heart in producing more protein during the conditions of ischemia is also not plausible.

Hence, the present study is the first to show that the cardioprotective effect induced by AT_1R blockade is associated with an upregulation of AT_2R protein content. This upregulation might involve Ang II binding to AT_2R. However, binding of Ang II with AT_2R will also activate AT_2R. Previous studies have shown that AT_2R activation induces apoptosis [53]. On the other hand, evidence also exists that AT_2R activation also increases cGMP [79-82, 101], a factor associated with cardioprotective effect. Hence, further analysis was carried out where apoptosis was examined in Langendorff preparations, and cGMP and PKC_ε were investigated in the hearts perfused in working mode.

Effects of AT_1R Blockade on Apoptosis after IR

Several studies have concluded that AT_2R mediate apoptosis via activation of mitogen-activated protein kinase phosphatase-1, dephosphorylation of Bcl_2 and upregulation of bax [53]. AT_2R activation would therefore be expected to increase apoptosis. In the present study, an interesting phenomenon was observed. CAN administration for 5 min before IR significantly reduced cardiomyocytes apoptosis with no corresponding change in post-ischemic recovery of mechanical function. Conversely, no change in cardiomyocytes apoptosis in combination with improved post-ischemic functional recovery was detected when the protocol entailed 40 min of CAN pretreatment before ischemia. Besides the length of CAN pretreatment, which might have affected the extent of apoptosis, no other mechanistic explanation has been found as yet.

Apoptosis or programmed cell death, has been suggested to play a critical role in the pathophysiology of cardiovascular disease and might contribute to ventricular damage, mechanical dysfunction and remodeling after MI [70]. Because of the large

number of activators and inducers involved in the pathway, apoptosis was thought to be a mechanism intrinsic to chronic ailments [70]. However, recent evidence have shown that apoptosis is involved in acute IR and has been suggested to contribute to IR injury [71].

Studies have shown that ischemia for 45 min followed by 2.25 hr of reperfusion can trigger apoptosis [109]. Apoptosis can also be detected in hearts exposed to 15 min ischemia and 1.5 hr of reperfusion [169, 170]. Since, apoptosis is an active, energy-requiring process, availability of ATP during longer durations of reperfusion might be an important determinant of the extent of apoptosis. However, the present study investigated apoptosis after 40 min of reperfusion. The main aim of this part of the thesis was to examine the relation of the extent of apoptosis to functional recovery in CAN-pretreated rat hearts. It was observed that CAN-pretreated rat hearts although increased the post-ischemic functional recovery, apoptotic indices were not changed. Therefore, it was concluded that apoptosis might not be significantly contributing to the injury in acute IR. Pretreatment of CAN had no effect on apoptosis; however, post-ischemic recovery of mechanical function was improved.

Effects of AT_1R Blockade and AT_2R Activation on cGMP Production after IR

Studies in different (aortic and coronary) vascular beds have shown that AT_2R activation causes increases in kinin, NO and cGMP levels [79-82, 101]. However, when the role of AT_2R was explored in myocardial studies, the possible involvement of NO or cGMP was not investigated [82, 100]. Although, an AT_2R-mediated increase in NO by bradykinin was supported by the myocardial studies, the downstream effector of bradykinin was not examined [100]. We report here, for the first time in the isolated working rat heart setting,

that CAN-pretreated hearts show an elevated level of cGMP, which was attenuated by concomitant perfusion with the AT_2R antagonist, PD. This confirms that AT_2R activation results in increase cGMP content that might be involved in eliciting the cardioprotective effect of AT_2R activation. Since no change in coronary flow was detected, cGMP might have mediated its beneficial effect directly on the myocardium. To that end, studies have shown that cGMP exerts an effect on myocardial tissue via cGMP protein kinase-mediated (1) modulation of sarcolemmal calcium influx, (2) reduction in myofilament calcium sensitivity, (3) altered sarcoplasmic reticulum function, (4) changes in action potential, (5) modulation of cell volume, and (6) reduction of oxygen consumption (as reviewed in references 120). Collectively, these events increase myocardial efficiency and thus preserve the myocardium from IR injury. Hence, increased myocardial cGMP content was correlated with the cardioprotective effect associated with CAN-mediated AT_2R activation.

One of the mechanisms for the AT_2R-mediated increase in cGMP is via NO production. Studies have shown that activation of AT_2R precipitates increases in nitrite and nitrate levels in coronary microvasculature [79]. Moreover, vascular studies looking at the effects of AT_2R have shown that activation of AT_2R results in an increase in cGMP production, which was abolished by NOS inhibitors [101]. Furthermore, additional studies in the myocardium show that activation of AT_2R and its subsequent cardioprotective effect was abrogated by NOS inhibition [82]. Since it has been established in vascular studies that an AT_2R-NO-cGMP pathway exists and this has been partially extended to myocardial studies [82, 100] where AT_2R-NO pathway operates, it can be hypothesized that

in isolated working rat heart, the AT_2R-mediated cGMP increase might have involved NO production.

Despite the evidence presented here, it is possible that AT_2R might increase cGMP via a NO-independent pathway. Studies with ANF receptors have shown that ANF can directly regulate cGMP. This is achieved by a direct regulation of membrane bound guanylate cyclase by ANF receptors such that NO is not required [171]. The possibility also exists that perhaps cAMP might be involved in cGMP regulation. In this context, studies have shown that cAMP can regulate cGMP levels via negative cross-talk [172]. However, the current evidence of regulation of cGMP directly or indirectly (via cAMP) by AT_2R is lacking. In contrast, numerous studies in vascular tissues have shown that AT_2R does mediate increase in NO and this NO is involved in the increase in cGMP content. Thus, it is likely that the AT_2R-mediated increases in cGMP content involves NO in myocardial tissue, however, experiments to identify NO as a downstream molecule of AT_2R-mediated cGMP increase in myocardial tissue are clearly warranted.

Effects of AT_1R Blockade and AT_2R Activation on PKC_ε Activation after IR

PKC_ε is perhaps one of the best studied intracellular signaling molecules and has been associated with ischemic preconditioning-induced cardioprotection (IPC or ischemic preconditioning is a phenomenon where one or more brief periods of ischemia with intermittent reperfusion protects the heart against a subsequent sustained period of ischemia) [112]. Numerous studies have implicated PKC_ε in the beneficial effects of both early and late preconditioning [174, 175]. Despite the identification of PKC_ε as a potential

mediator of IPC-mediated cardioprotection, pathways leading to PKC_ε activation have not been clearly elucidated.

Recently, a new possible pathway for PKC_ε has been suggested involving NO [125]. Preliminary studies have suggested NO involvement in triggering late preconditioning (PC) [173]. Experiments done in an *in vivo* canine model of regional ischemia showed the beneficial effects of PC on IR arrythmias was attenuated by L-NAME (a NO synthase inhibitor) and methylene blue (a guanylate cyclase inhibitor). Moreover, L-NAME abolished the reduction in ischemic arrhythmias induced by intracoronary bradykinin in the same canine model suggesting the bradykinin may be a prime mediator of NO-induced PC. Recent evidence has highlighted NO in the delayed form of PC, also where the cardioprotection is evident at 24-72 hours after initial PC [173].

Moreover, in the same model of PC, it was established that PKC_ε is activated. Bugge and Ytrehus [174] found that the PKC antagonists, polymyxin B and chelerythrine, blocked the protective effect of IPC. Furthermore Mitchell *et al.* [175] showed that IPC, induced by a 2 min transient ischemia and 10 min of reperfusion, significantly increased functional recovery at 40 min after 20 min of global no-flow ischemia. The PKC inhibitors, chelerythrine and staurosporine, abolished this protective effect, while the diacylglycerol analog, 1-steroyl-2-arachidonyl glycerol, mimicked the benefit. Moreover, immunoblots for PKC isoforms show the PKC_ε was translocated to the particulate fraction, suggesting the role of PKC_ε in cardioprotection [175].

However, later studies done by Ping *et al.* [68] pooled this concept together and showed that PKC_ε is perhaps activated by NO. To that end, they made four pertinent observations: (1) administration of L-nitro-arginine completely blocked the activation of

PKC_ε; (2) administration of two structurally dissimilar NO donors increased the translocation of PKC_ε to particulate fraction, indicating the activation of PKC_ε; (3) chelethyrine blocked this increased activation of PKC_ε by NO donors; and (4) chelethyrine also blocked the NO donor induced late preconditioning effect on myocardial stunning and infarction. Taken together, these findings suggest that PKC_ε plays an integral role in NO-mediated delayed cardioprotection.

Since AT_2R activation is known to increase NO levels and previous studies from our laboratory showed an increase in PKC_ε content during cardioprotection induced by AT_1R blockade [176] (an event also observed by Ping *et al.* in the rabbit IPC model previously) it was hypothesized that AT_1R blockade would increase PKC_ε activation via AT_2R. When the particulate fraction of PKC_ε in myocardium was examined as an index of activation, CAN was found to increase PKC_ε activation. This increase was subsequently inhibited by PD administration, suggesting AT_2R activation is involved in mediating PKC_ε activation. Cumulatively, this suggests that in addition to AT_2R-mediated increase in NO and cGMP, AT_2R is also engaged in (NO-mediated) PKC_ε activation, and this might be another mechanism of therapeutic action exerted by AT_2R. In support, previous studies have shown that AT_2R is involved in PKC activation [84, 91]. However, the lack of specific isoform of PKC being examined in that study, the avian origin of cardiomyocytes and no inferences on the potential cardiac role of this pathway seriously undermined the importance of this finding. Thus, it can be suggested that AT_2R mediates some of its therapeutic effects through PKC_ε. This might involve mitochondrial K_{ATP} channels as a possible therapeutic route, as a recent study implicated mitochondrial K_{ATP} as a possible downstream effector of PKC_ε-mediated cardioprotection [177].

Hence, taken together, the present study suggests that: (a) CAN mediates some of its therapeutic effect by AT_2R activation in acute IR; (b) this activation involves locally generated Ang II; (c) AT_2R mediates some of its beneficial effect by increased cGMP; and (d) some degree of the cardioprotection is also conferred by PKC_ε activation.

Mechanism Proposed

During IR, Ang II levels in the intersitial space increases. Since AT_1R is blocked by CAN, most of the Ang II is diverted towards AT_2R. The binding of Ang II to AT_2R stabilizes the Ang II-AT_2R such that AT_2R protein content is increased. This binding of Ang II to AT_2R also initiates AT_2R activation, which results in increased bradykinin level. This increased bradykinin in turn increases NO which results in increased production of cGMP and activation of PKC_ε. The possibility exists that cGMP might be involved in PKC_ε activation. However, studies in pituitary gland showed that cGMP increase does not influence PKC_ε activation. Hence, NO might be the upstream molecule for PKC_ε and cGMP (Figure 25).

LIMITATIONS OF THE STUDY

Despite some of the pertinent findings of this thesis, including isolation of possible pathways involved in cardioprotection via AT_2R, there are problems that impose limitations to this study. Firstly, these studies have presented indirect evidence of a possible role of cardiac Ang II. However, no actual determination of Ang II in support of this claim was made. Evidence of actual presence of Ang II during IR is lacking. Initial studies by others have substantiated that isolated heart has the capacity to synthesize Ang II. To that end, Lindpaintner *et al.* [15] has shown in an isolated beating rat heart, angiotensinogen, a precursor and rate-limiting molecule of the Ang II pathway, is synthesized locally and in fact contributes to most of Ang II cardiac pool in the later part of the perfusion. One study has actually measured Ang II in IR, however the measurement was done in the myocardium. No actual determination of Ang II release into the perfusate was ever done.

Secondly, the present studies were done using an *in vitro* system which precludes extrapolation of these results to *in vivo* settings. However, *in vitro* studies are specifically designed to observe the effects of one system to a given insult. This enables researchers to identify, isolate and find possible solutions to a factor or a system under investigation. Thus, results from the *in vitro* system, although lacking closeness to the *in vivo* clinical setting, still have important clinical implications.

Thirdly, one of the explanations provided for the increase in AT_2R content is a decreased degradation rate of AT_2R. Although, this thesis has discussed sufficient reasons to indicate that increased expression of AT_2R protein was not involved,

determination of mRNA levels would have put the issue at rest. However, determination of mRNA levels of AT_2R protein could be a subject of future research.

It is also becoming evident that perhaps assessment of apoptosis by TUNEL-assay might not be sufficient. In this respect, studies published in apoptosis are looking at this phenomenon by employing two different techniques. Although, TUNEL assay is a sensitive assay and is a good indicator of cells undergoing apoptosis, reinforcement of the results with another technique might have strengthened the data set.

This study has isolated beneficial effects of AT_1R blockade that are mediated by AT_2R activation. Although activation of AT_2R increased cGMP content and PKC_ε activation were detected, whether cGMP and PKC_ε are directly involved in cardioprotection under these conditions still remains to be determined. This could be assessed by the use of a selective inhibitors of cGMP or PKC_ε in the presence of an AT_1R antagonist. Furthermore, the relative contribution of cGMP and PKC_ε in cardioprotection remains to be elucidated.

Moreover, this thesis has found cGMP and PKC_ε increase in CAN-pretreated rat hearts. It has been proposed that perhaps NO is the common molecule involved in precipitating increase and activation of cGMP and PKC_ε, respectively. Although previous studies have shown NO-mediated cGMP production [74, 101, 153-155] and PKC_ε activation [68], no direct cause-effect determination of this result was ever made during the present study. Future experiments on the subject of potential NO involvement in the regulation of cGMP and PKC_ε are clearly warranted.

Inspite of these limitations, this thesis has highlighted and found some of the important mechanisms involved in AT_2R-mediated cardioprotection and perhaps would be a subject of interest for future investigation and therapeutic endeavors.

FUTURE CLINICAL IMPLICATIONS

Initial studies done with Ang II were largely concentrated and geared towards the understanding of AT_1R. However, recent evidence of the reappearance of AT_2R in various cardiac pathologies has generated a lot of interest in this receptor subtypes [23] and became the focus of the laboratory. While conflict exists as to what the precise role of AT_2R is (i.e., is it deleterious because of apoptosis or beneficial because of generation of bradykinin, NO,and cGMP), the concept that AT_2R is a potentially beneficial receptor is becoming increasingly important and gaining acceptance. Despite the development of its ligands in 1990s, knowledge of its signal transduction pathways still remains incomplete. This thesis specifically addressed the role of AT_1R blockade in the context of AT_2R modulation and has tried to identify some of the transduction pathways involved. This thesis has isolated and extended some new pathways and therefore should add to the understanding of AT_2R as a potential protective receptor. However, the mechanism behind AT_2R cardioprotection is still far from being fully elucidated. Hopefully, these findings may add to the existing knowledge of AT_2R signal transduction mechanisms and might lead to the development of beneficial drugs for various cardiac pathologies. Since AT_2Rs are elevated during different cardiac diseases, future clinical studies might harness this increase in AT_2R. To that end, a combination of AT_1R antagonist and AT_2R agonist as a potential therapeutic cocktail may need to be considered in future for Ang II precipitated pathologies.

CONCLUSION

In patients with heart failure, neurohumoral systems such as the sympathetic nervous system and the RAS are stimulated to compensate for the cardiac dysfunction [178]. However, the persistent activation of RAS may cause deterioration of heart failure because of its vasoconstrictive, volume-retaining and mitogenic actions [167]. Initially, treatment with ACEI has been shown to decrease morbidity and mortality in patients with heart failure [14]. However, ACEI do not only suppress the RAS but also inhibit bradykinin breakdown. Although, bradykinin plays a role in cardioprotective mechanism, this factor may evoke undesirable side-effects, such as dry cough and angioedema.

Recently, specific inhibitors of RAS, such as AT_1R antagonists, have been developed. Although the majority of the studies carried out with losartan (the first orally non-peptidic available antagonists) showed no effect [119], some investigations showed protective [116-118] and deleterious effects [132, 133] as well. The present study was designed to evaluate, in particular, the effects of the AT_1R antagonist, CAN, in prevention of acute IR cardiac dysfunction. To that end, pretreatment with CAN for 40 min prior to IR insult increased post-ischemic functional recovery in Langendorff perfused hearts. This was further associated with increased AT_2R protein content detected by western immunoblot. Moreover, analysis of apoptosis showed no significant change in any parameters indicating the dissociation of apoptosis from the post-ischemic functional recovery.

These findings were taken one step further, and the phenomenon was analyzed in the isolated working rat heart model. In this set-up, questions related to activation of different signal transduction molecules downstream of AT_2R were also addressed. This

study showed a significant increase in post-ischemic functional recovery by CAN, although, no change was found with losartan. Changes in molecular parameters were less pronounced with losartan when compared to CAN. When PD was co-administered with CAN, there were attenuated effects on post-ischemic functional recovery, cGMP levels and PKC_ε activation. Analysis of AT_2R, on the other hand showed an increase in AT_2R protein which was in agreement with the previous findings in Langendorff hearts. Cumulatively, these studies show that there is no difference in terms of the effect of CAN on acute IR as both models showed the same cardioprotection and increase in AT_2R protein content. Secondly, pretreatment of CAN causes an increase in AT_2R protein levels and AT_2R were subsequently found to be activated. Thirdly, this activation of AT_2R was correlated with increase in cGMP levels and PKC_ε activation, two signal transduction molecules of cardioprotective importance; however, no change in apoptosis was observed. Moreover, indirect evidence suggests that Ang II is formed in myocardium, under acute conditions, and this Ang II might be involved in AT_2R activation.

Hence, taken together, AT_1R blockade with CAN makes Ang II more accessible to AT_2R. This results in activation and inadvertent increase in AT_2R protein content. Activated AT_2R triggers pathways leading to cGMP and PKC_ε activation, possibly by NO. However, activation of AT_2R does not trigger apoptotic pathways. Combination of all these events may be involved in the cardioprotective effect of CAN in acute IR.

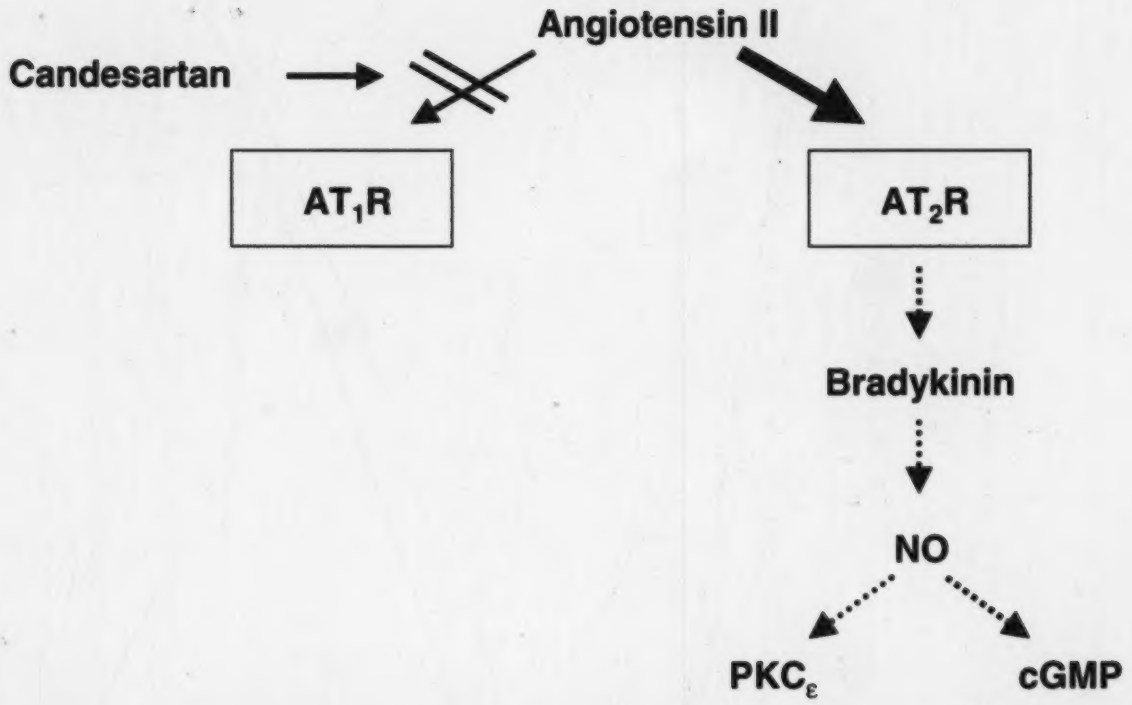

Figure 25 Proposed pathway of candesartan-mediated cardioprotective effect. Blocking of angiotensin II type 1 receptor (AT_1R) causes a shift in increased angiotensin II towards angiotensin II type 2 receptor. Activated angiotensin II increases bradykinin levels which in turn increases nitric oxide (NO). This increase in NO increases cyclic guanosine monophosphate levels (cGMP) and activates protein kinase C-epsilon (PKC_ε). Dashed arrows represents the proposed pathways.

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